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Centrifugal Precipitation Chromatography - a Powerfull Technique for the Isolation of Active Enzymes from Tea Leaves (*Camellia sinensis*)

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Abstract

Centrifugal precipitation chromatography (CPC) was developed approximately 10 years ago. In contrast to other counter-current chromatographic techniques, the CPC system is operated with two mutually miscible solutions separated by a cut-off membrane. CPC was firstly introduced for the separation of proteins using an ammonium sulfate gradient. In this study we describe a novel approach using solvent based protein precipitation for the isolation of active plant enzymes from tea leaves (*Camellia sinensis*) by CPC. We developed a gradient based on acetone and Tris-buffer, because the biological activity of carotenases in tea leaves cannot be preserved in the presence of ammonium sulfate. Parameters such as the critical solvent concentration, flow rate, buffer concentration, and sample load were determined and/or optimized. Subsequently, the newly developed separation protocol was successfully used for the isolation of active carotenoid cleavage enzymes from tea leaves. The isolated enzymes showed high enzymatic activities and purities and could be directly used for enzymatic assays and structure elucidation.

Keywords

CPC; CCD; Carotenoid Cleavage; Tea; *Camellia sinensis*

Introduction

The interest in carotenoid cleavage enzymes, involved in the biosynthesis of volatile norisoprenoids, dramatically increased over the last decade because norisoprenoids are widely distributed in nature and contribute to the quality of food. Of particular interest is the formation of norisoprenoids, like β -ionone or damascenone, which possess very low odor thresholds. Furthermore, the importance of carotenoid derived aroma compounds in Japanese green tea has been investigated by many research groups. During tea processing the total loss in carotenoids is approximately 40% and many volatile compounds derived from carotenoid precursors have been identified in tea [1,2]. Besides thermal degradation various enzymes, such as polyphenoloxidase, lipoxygenases, and β -carotene bleaching enzymes are also

involved in the unspecific formation of norsioprenoids in tea [1]. Recently we could elucidate that specific carotenoid cleavage enzymes are involved in the formation of C₁₃ norisoprenoids in tea leaves (*Camellia sinensis*) during processing, as well as in commercially available tea (Figure 1) [3]. Enzymes obtained from spring and autumn tea showed similar kinetic properties, but are characterized by immense variations in the isoelectric points or molecular masses, depending on the harvesting season [3]. Whereas the tea leave carotenoid oxygenases harvested in autumn have an acidic isoelectric point, enzymes from spring tea have an isoelectric point at neutral pH. In previous studies carotenoid oxygenases from natural tissues were successfully purified by matrix-free isoelectric focusing [3,4,5,6]. This technique was a very effective because those enzymes are characterized by acidic isoelectric points (Quince fruit 2.8, Star fruit 3.5, Nectarines 3.6) and therefore the target enzymes can be separated from many contaminating plant proteins [4,5,6]. In case of carotenases from spring tea this technique was less helpful because the majority of plant proteins has isoelectric points around pH 7. The application of centrifugal precipitation chromatography, which separates proteins based on their solubility in ammonium sulfate or organic solvent, provides an excellent alternative purification method.

Experimental

Reagents

The solvents and reagents used for the preparation of the crude enzyme extracts were of analytical grade and purchased from (Cica, Kanto Chemicals, Japan). Acetone and water (HPLC-grade) used for CPC separations were from Fisher Scientific (Fair Lawn, NJ). The buffer salts (Tris-Base, Potassium Chloride, Magnesium Chloride) were purchased from Sigma (St. Louis, MO); and the SDS-PAGES, electrophoresis buffer, and protein marker were from Invitrogen (Corporation, Carlsbad, CA).

Equipment

CPC—The preparative separation was performed on a compact seal-free mini-centrifuge (Prototype, Pharma-Tech Research, Baltimore, MD, USA) CPC system [7]. The capacity of upper and lower channel was 4 mL and 8 mL respectively. The membrane (Spectrum Laguna Hill, CA), sandwiched between the upper and lower disk to form two channels, had a molecular cut-off of 6000-8000 Da. The upper channel was connected to a Perkin Elmer (Perkin Elmer, Norwalk, CT, USA) HPLC pump Model 200 series and the lower channel to a SCL 10A, LC AD Shimadzu (Shimadzu, SCL-10A and LC-10AD, Shimadzu, Kyoto, Japan) gradient pumping system. The fractions were collected with a LKB Bromma 2211 superfrac fraction collector (Pharmacia, Stockholm, Sweden).

Photometer—()

Electrophoresis—The isolated proteins were analyzed using an Invitrogen X-Cell Sure Lock Mini-Cell connected to a BIO-RAD (BIO-RAD, Life Science Research, Hercules, CA) Power Pac 300.

Plant material

All tea leaves used for our experiments were picked from *Camellia sinensis* shrubs at the National Institute of Vegetable and Tea Science, Kanaya, Shizuoka prefecture, Japan, in spring 2006. The leaves were stored at 4°C until the initial preparation steps were carried out.

Preparation of crude enzyme extracts

Eighty gram of tea leaves were transferred into 120 mL chilled buffer (125 mM KCl, 100 mM Tris-base, 5 mM MgCl₂, adjusted with HCl to pH 7) and homogenized in a Waring Blender. The insoluble plant residues were separated by sedimentation centrifugation at 6000 g for 10 min. The proteins dissolved in the supernatant were precipitated with 95% acetone at 0°C for 2 hours. The resulting protein precipitate was separated from the acetone by filtration (0.2 µm), rediluted in water, frozen, and lyophilized to obtain a stable dry protein powder. For determination of the enzymatic activity in relation to the acetone concentration proteins were precipitated with 70%, 75%, 80%, 85%, and 90% acetone relative to the supernatant after centrifugation.

Determination of the critical solvent concentration

The acetone powder was dissolved in Nanopure water and for the protein precipitation 200 µL aliquots were distributed into vials and stored on ice. Chilled acetone was added in small portions; at the beginning every 10 min 100 µL and afterwards every 5 min 200 µL until 18%, 36%, 54%, 72%, and 90% final solvent concentrations were reached. For a complete precipitation the vials were sealed and stored over night at -20°C. After temperature adjustment to 4°C the precipitated proteins were separated by sedimentation centrifugation. The amount of proteins remaining in the supernatant was determined using a modified Bradford assay. The dye solution was obtained by dissolving 25 mg of Coomassie Brilliant Blue in 12.5 mL ethanol (96% v/v). Afterwards 25 mL of phosphoric acid (85% w/v) were added and the final volume of 250 mL (volumetric flask) was made up with Nanopure water. For each assay 100 µL of sample solution was mixed with 1 mL of filtered dye solution and vortexed carefully. After 5 min the absorption at 595 nm was measured. As blank 100 µL of Nanopure water instead of sample were added to the dye solution.

Determination of enzymatic activity

The carotenoid cleavage ability of the isolated enzymes was monitored at 505 nm, using β-carotene as substrate [4]. The assay mixture, containing CPC output-fraction (enzyme solution) and β-carotene (initial concentration 9.8 µmol L⁻¹) were incubated at 60°C for 1 h. The difference in absorbance before and after incubation was calculated as relative enzymatic activity, setting the initial concentration to 1.

Determination of the osmosis rate between sample and solvent channel

The osmosis rate between the channels was tested by running a gradient of acetone-Tris-buffer. The output volume was measured with a small measuring cylinder. The following equation was used for the calculation of the osmosis rate:

$$\text{osmosis rate} = 1 - \frac{\text{output volume}}{\text{input volume}}$$

After evaporation of the solvent, the salt concentration in the CPC output fractions was determined gravimetrically. The salt output was calculated in relation to the salt input, which was calculated from the salt content of the Tris-buffer (60 mL h⁻¹ of 125 mM KCl, 100 mM Tris-base, 5 mM MgCl₂, adjusted with HCl to pH 7):

$$\text{salt concentration of output fraction} = \frac{\text{output salt concentration}}{\text{input salt concentration}}$$

Sample preparation for CPC

0.5 g of protein powder was dissolved in 25 mL of Nanopure water and heated at 90°C for 30 min. The thermostability of carotenases from tea leaves (active during and after the tea manufacturing process [3,8]) preserves the enzymatic activity, whereas the conformation of other proteins is intensely changing under these conditions, resulting in denatured protein agglomerates. The precipitates were removed by sedimentation centrifugation at 10000 g for 20 min. Afterwards the samples were desalted and concentrated with a 10 kD filter unit from Centricon (Biocompare, South San Francisco, CA 94080) until a final volume of 1 mL was reached. A second sedimentation centrifugation at 10000 g for 10 min was necessary to remove all insoluble particles.

CPC separation

The CPC apparatus was filled with acetone. After injection the sample channel was eluted from the inner to the outer terminal pumping Tris-HCl buffer (100 mM Tris-HCl, 125 mM KCl, 5 mM MgCl₂, pH 7) with a flow rate of 0.1 mL min⁻¹ throughout the sample channel. The rotation speed of the separation disc was set to 1000 rpm. The experiment was started with an isocratic step of 100 % acetone for 120 min. The flow rate inside the solvent channel was set to 1 mL min⁻¹. Subsequently, the proteins were eluted by running a linear gradient to 0% acetone within 600 min. Finally, the elution was finished by an isocratic step using 100% Tris-HCl buffer, maintained until 1080 min, in the solvent channel. Fractions were collected in intervals of 60 min at the sample channel output, evaporated to dryness, and dissolved in equal volumes of Nanopure water (1.8 mL).

Purity analysis of isolated enzymes by SDS-PAGE

Before sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) the samples were concentrated and desalted with a 10 kDa filter unit from Centricon. 20 µl of the concentrated sample solution were mixed with 10 µL NuPAGE LDS sample buffer 4× (Invitrogen) and after mixing heated for 10 min at 90°C. The negatively charged protein/SDS complexes were separated on a NuPAGE 12% Bis-Tris Gel (Invitrogen) at a constant voltage of 70 V. The molecular weight was calculated based on the R_f values of the Mark12 Unstained Standard (Invitrogen).

Results and Discussion

Critical solvent concentration

The suitability of an organic solvent for CPC separations can be tested by the determination of the critical solvent concentration which provides information about the concentration necessary for the precipitation of proteins from the crude extract. Therefore, the crude protein extract of tea was diluted in Nanopure water and the constituents precipitated with acetone. The protein concentration of the supernatant was determined by a modified Bradford assay. The absorption of the assay mixture measured at 595 nm is plotted in Figure 2a. During a CPC run, all proteins are initially precipitated with ammonium sulfate or solvent. As shown in Figure 2a no proteins could be detected in the supernatant after acetone precipitation using solvent concentrations above 60% by a modified Bradford assay, with a detection limit of approximately 0.1 µg mL⁻¹. Furthermore, acetone was chosen because of its ability to preserve the desired enzymatic activity. The enzymatic activity of crude protein extracts, using acetone concentrations between 70% and 90%, was measured (Figure 2b). The highest relative activity could be assayed using 80% acetone for the precipitation step. In conclusion the target enzyme should elute from the CPC at an acetone concentration of approximately 80% with good separation from many contaminants, which are precipitated at acetone concentrations below

60% (results are discussed in the paragraph isolation of carotenoid cleavage enzymes from tea leaves).

Optimization of the gradient

The number of references using solvent gradients for the CPC separations is still limited [9, 10] and so far no CPC application for the isolation of active enzymes with organic solvents was available. Furthermore, separations using ethanol as precipitation agent were tested and established [10], but no data for the solvent acetone were available. Therefore, an optimization of separation parameters was necessary. Studies on the acetone transfer and the osmosis rate through the membrane were performed without sample injection, but otherwise equal experimental conditions.

First experiments using acetone and water showed that the cross over between the sample and solvent channel was too high. A similar effect was described for water-ethanol gradients [10]. A significant difference between the flow rate in the water channel and the solvent channel ($10\times$) led to a decrease of the output flow rate of up to 75%. For the dextran fractionation, this problem was overcome by increasing the flow rate in the water channel [10]. In addition the cross over between both channels is influenced by density defined as mass/volume ratio. Buffer salts increase the volume only by a low factor; however the mass increases by a higher factor leading to an increase in density. In order to enhance the density, water was replaced by Tris-buffer. The replacement persuades the migration between both channels and additionally stabilizes the enzymatic activity. If no centrifugal force field is applied, an exponential change of the osmosis rate can be expected [11], but the actual processes are influenced by rotation, counter flow, and pressure. Under our experimental starting conditions nearly all liquid migrated through the membrane. Subsequently the osmosis rate decreased until 12 h, and then remained nearly stable between 14–18 h (Figure 3 squared line). As expected the osmosis rate from the sample to the solvent channel was found to decrease with increasing buffer concentrations in the solvent channel. Under consideration of the output salt concentration (Figure 3 dotted line) and the liquid transfer (Figure 3 squared line) only the water migrated from the sample to the solvent channel, but no salt (Figure 3 dotted line, 1–3 h). With increasing buffer concentrations in the solvent channel the remaining salt eluted within the output fractions 4–7. Afterwards the salt content increased slowly (Figure 3 dotted line), whereas in parallel the osmosis rate decreased (Figure 3 squared line, 7–12 h). During the final isocratic step, when only buffer was flowing through both channels, the buffer salts partly migrated from the solvent channel into the sample channel (12–18 h). This effect was due to the different flow rates inside the sample and solvent channel. Whereas the centrifugal force was the same, the flow rate in both channels differed by a factor of 10. The retention period of the liquid in the sample channel was longer and therefore the resulting pressure lower, which lead to an enrichment of the buffer salts. These effects, especially the migration during the initial gradient step (100% acetone in the solvent channel) promotes the protein precipitation, which is essential for CPC separations.

Isolation of carotenoid cleavage enzyme from tea leaves

The pre-purified samples (details are given in the experimental section) were subjected to centrifugal precipitation chromatography. The separation conditions were used as described in the previous paragraph. Figure 4 shows the activity plot of data obtained from tea samples harvested in spring. The enzymatic activity was screened using β -carotene, one of the major carotenoids in tea, as substrate [11,8]. The highest activity could be determined in fraction 3. The dashed line (Figure 4) shows the calculated acetone concentration inside the sample channel. Active fractions elute at acetone concentrations lower than 90% calculated solvent concentration, which is in accordance to the results shown in Figure 2b, where the highest enzymatic activity could be detected using 80% acetone for protein precipitation.

Shortly after the introduction of centrifugal precipitation chromatography a systematic study about the shear stress and the precipitation effects was carried out. The recovery of α -chymotrypsin was 89% using ammonium sulfate as precipitation agent [13]. Based on this study and the knowledge that liquid-liquid chromatographic techniques are suitable for the isolation of a wide range of bioactive compounds [summarized by 14,15, and 16] we expected that the activity of our target enzyme could be preserved. In the present study we obtained relative activities of more than 60% (Figure 4, fraction 3). Purification of the same crude enzyme extract by matrix-free isoelectric focusing or size exclusion chromatography yielded in lower relative activities. Therefore, we could confirm that centrifugal precipitation chromatography is suitable for the isolation of active plant enzymes. Furthermore CPC could be a powerful tool for the isolation of recombinant enzymes. In many studies recombinant enzymes are not isolated from their host cells because the decrease in enzymatic activity and sometimes low yields. For functional characterization the genes encoding enzyme information are heterologously co-expressed with genes encoding substrate information also throughout many studies for the functional characterization of carotenoid oxygenases [17,18,19,20,21, and 22].

Purity determination by SDS-PAGE

After we demonstrated that enzymatic activity can be preserved we were also interested in the purities of the obtained fractions. Based on the results of the critical solvent concentration and the function of enzymatic activity in relation to the acetone concentration (Figure 2a and 2b) high purity of the early active CPC fractions (Figure 5) could be expected. The SDS-PAGE analysis of selected CPC fractions confirmed our expectations. The molecular size of the major protein of approximately 69 kDa in fraction 3 confirmed the previous results, where we could show that enzymes of the same molecular size (66 kDa) are responsible for specific carotenoid cleavage in tea leaves [3]. The difference of 3 kDa can be easily explained with the limited accuracy of the mass determination by SDS-PAGE. The activity detected in later fractions is most likely related to the 48 kDa protein [3].

Conclusions

Our presented results show that CPC is an excellent tool for the isolation of active plant enzymes from crude extracts. We could demonstrate that the isolation of enzymes under optimized separation conditions leads to active enzymes of high purity.

Acknowledgments

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Figure 1.

Enzymatic oxidation of β -carotene by specific carotenoid cleavage oxygenases in tea leaves

Figure 2.

(a) Acetone concentrations used for protein precipitation in relation to protein content (measured by Bradford Assay) of the supernatant (unprecipitated proteins).

(b) Carotenoid cleavage ability of a crude enzyme extracts obtained after precipitation with different acetone concentrations.

Figure 3.

Osmosis rate and salt concentration in output fractions (sample channel) of the solvent system acetone-Tris-buffer, sample channel: Tris-buffer at a flow rate of 0.1 mL min^{-1} and solvent channel: gradient of acetone-Tris-buffer 0-2 h 100 % acetone, 2-12 h 100 – 0 % acetone, 12-18 h 100 % Tris-buffer at a flow rate of 1 mL min^{-1} and 1000 rpm.

Figure 4. Activity profile and calculated solvent gradient of a CPC separation (Separation conditions as given in the legend of Figure 2).

Figure 5. SDS-PAGE (NuPAGE 12 % Acrylamide) stained with SilverXpress Staining Kit. The numbering is equally to the CPC fraction number in Figure 3 - (A) 3,(B) 5,(C) 6,(D) marker, (E) 10, (F) 14, (G) 17.