Application of centrifugal precipitation chromatography and high-speed counter-current chromatography equipped with a spiral tubing support rotor for the isolation and partial characterization of carotenoid cleavage-like enzymes in Enteromorpha compressa

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Application of centrifugal precipitation chromatography and counter-current chromatography equipped with a spiral tubing support rotor for the isolation and partial characterization of carotenoid cleavage-like enzymes in *Enteromorpha compressa* (L.) Nees

Susanne Baldermann[1], Andriati Nigrum Mulyadi[1], Ziyin Yang[1], Ariaka Murata[1], Peter Fleischmann[2], Peter Winterhalter[2], Martha Knight[3], Thomas M. Finn[3], Naoharu Watanabe[1]

[1]*Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*

[2]*Institute of Food Chemistry, Technische Universität Braunschweig, Schleinitzstrasse 20, 38106 Braunschweig, Germany*

[3]*CC Biotech LLC, 9700 Great Seneca Highway, Rockville, MD 20850*

Running title:

Counter-current chromatographic techniques for the isolation of enzymes

Correspondence:

Susanne Baldermann

Laboratory of Natural Products Chemistry, Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan, TEL & FAX 81-(0)54-238-487

Abbreviations

ATPS - Aqueous two-phase solvent system, CCD – Carotenoid cleavage dioxygenase, spCCC - counter-current chromatography system equipped with a spiral tubing support rotor, SPME - solid phase micro-extraction

Keywords

CCD, Centrifugal precipitation chromatography, Protein, Seaweed, Spiral tubing support rotor, spCCC

Abstract

Centrifugal precipitation chromatography and a high speed counter-current chromatography system equipped with a spiral tubing support rotor (spCCC) were successfully applied for the identification and isolation of carotenoid cleavage-like enzymes from *Enteromorpha compressa* (L.) Ness. This is the first study separating active enzymes from a complex natural matrix by spCCC. The target enzymes were identified after fractionation of the proteins in an acetone Tris-buffer gradient by centrifugal precipitation chromatography. Also, an aqueous two-phase solvent system (ATPS) consistent of PEG 1000 and mono- and dibasic potassium phosphate was used for the isolation of the enzymes by spCCC. The purified fractions contained two proteins of 65 and 72 kDa, respectively. The enzymes could cleave β-carotene and β-apo-carotenal to produce β-ionone.

1 Introduction

A liquid-liquid partitioning method, counter-current chromatography, has been successfully applied for the isolation of a broad range of naturally occurring compounds [reviewed by 1-3]. This technique provides advantages over conventional column chromatography by eliminating the use of solid phase supports, which cause losses due to interaction with reactive surfaces of solid stationary phases and irreversible adsorption. Early on there was interest to apply counter-current chromatographic techniques for the isolation of proteins [4-6], however the instruments were mechanically complex and the separations were poor mainly due to the low retention of aqueous solvent systems. In the last decade new instrumentation has been developed, which is suitable for the separation of large bio-molecules, such as proteins, polymerized pigments, as well as RNA and DNA plasmids [7-9]. To increase the retention of stationary phases of aqueous solvent systems new counter-current chromatography rotors such as spiral disk assemblies or a spiral tubing support have been introduced [10, 11]. These have been successfully applied for separations of standard proteins and peptides with sufficient levels of stationary phase retentions using highly polar solvent systems [11-13].

Centrifugal precipitation chromatography separates molecules according to their solubilities in ammonium sulfate or other precipitants, such as organic solvents [14]. Previously, this instrument was successfully applied for the isolation of enzymes from *Camellia sinensis* with preservation of the biological activity [15].

Here, we will present our results of the isolation and partial characterization of a carotenoid cleavage-like enzyme of *Enteromorpha compressa* (L.) Nees applying the two novel techniques centrifugal precipitation chromatography and a high speed counter-current chromatography system equipped with a spiral tubing support rotor (spCCC). The number of chromatographic steps required for protein purification and the risk of sample loss and denaturation can be reduced by application of these techniques and in particular biological activities are maintained.

We are interested in formation of secondary metabolites derived from carotenoids, which can function as growth factors, hormones, signaling or aroma-active constituents. Volatile norisoprenoids, such as the potent flavor compound β-ionone, are produced by a diversity of algae taxa and contribute the aquatic odors, however little is known about their biosynthesis pathways. In the last decade there has been special interest in the isolation and characterization of carotenoid cleavage dioxygenases (CCDs) involved in the formation of aroma compounds derived from carotenoids. CCDs are a heterogeneous super family of polyene chain oxygenases ubiquitously distributed. CCD enzymes have been isolated from plant tissues [15-17] and have been identified and characterized using homology based cloning approaches [reviewed by 18]. By application of centrifugal precipitation chromatography and spCCC we could prove for the first time that functional enzymes of these families are present in the multi-cellular green alga *Enteromorpha,* known as "Aonori" (green laver) in Japan and consumed as seasoning.

2 Materials and Methods

2.1 Instruments

2.1.1 Centrifugal precipitation chromatograph

A bench-top centrifuge with a controller (Pharma-Tech Research Corporation, Baltimore, MD,USA, prototype) was used for these experiments. The centrifugal precipitation rotor (Machine Instrumentation, Design and Fabrication, National Institutes of Health, Bethesda, MD, USA) consisted of a dialysis membrane (#132675 Spectra/Por MW cut off = 6-8,000, Spectrum, Laguna Hill, CA, USA) sandwiched between two 1.5 cm high density polyethylene disks (15.2 cm OD) that have spiral grooves which form a spiral flow channel divided by the semi-permeable membrane as described previously [8]. The upper part of the channel, 1 mm high is where the sample flows in the diluted buffer provided by a SSI pump (State College, PA, USA). The gradient elution is pumped using a D-Star Dual Gradient Delivery System (Manassas, VA USA) through the lower half of the flow channel

which is 2 mm deep. The fractions were collected with a LKB Bromma 2211 Superfrac fraction collector (Pharmacia, Stockholm, Schweden).

2.2.2 High-speed counter-current chromatograph equipped with spiral tubing support rotor (spCCC)

The other instrument used in the present study is a J-type planet centrifuge manufactured by Centrichrom Inc., Williamsville, NY USA as described previously [11]. The spiral tubing support (STS) rotor, made by laser lithography, is a frame of circular channels which hold continuous tubing in interweaved spirals that are layered in the rotor and held in place with a plastic top. The fluorinated plastic tubing (1.6 mm ID, total volume 135 mL, FEP No. 14, Zeus Industrial Products, Orangeburg, SC, USA) is wound from the bottom to the top where it is connected to the flow tubing that enters the shaft and out the center axis of the instrument where it is clamped with the inflow tubing as described previously [12]. The centrifuge is run at 800 rpm at a flow rate of 1 mL min^{-1} provided by a SSI pump. A LKB Bromma 2211 Superfrac fraction collector is used to collect 3 min fractions.

2.2 Reagents and materials

The solvents and reagents used for the preparation of the crude enzyme extracts were of analytical grade and purchased from Cica (Kanto Chemicals, Tokyo, Japan). Acetone and water (HPLC-grade) used for centrifugal precipitation chromatography separations were from Fisher Scientific (Fair Lawn, NJ, USA). The buffer salts (Tris, postassium chloride, and magnesium chloride) were purchased from Wako (Shizuoka-Shi, Japan). Selected fractions were analyzed by SDS-PAGE on 12.5 % polyacrylamide gel (e-PAGE 12.5 %, Tokyo, Japan) using the Precission Plus Protein Dual Colour Standard (Bio-Rad, Tokyo, Japan) as the protein MW marker. The proteins were stained with Silver Staining Kit III (Wako, Japan) or Bio-Safe Coomassie Blue G-250 Stain (BioRad, Japan) following the manufacturer's instructions. Other reagents including PEG 1000, dibasic and monobasic potassium phosphate were purchased from Sigma (St. Louis, MO, USA).

The proteins were extracted from seaweed (*Enteromorpha compressa* sp.) grown in Lake Hamana , Shizuoka prefecture, Japan.

2.3 Preparation of crude sample

Protein extraction from most algae is difficult due to the presence of large amounts of anionic cell-wall polysaccharides which strongly hinder the solubilization of proteins during extraction procedures. There are several methods to increase the efficiency such as sonication, maceration with cellulose or macerozyme or pre-incubation with alcohols. In this study 350 g algal were pre-incubated with 70% ethanol for 30 min. The residue obtained by filtration (Durapore 0.45 µm, Millipore, Bedford, MA, USA) was homogenized in 250 mL chilled buffer (50 mM Tris–HCl, 125 mM KCl, 5 mM $MgCl_2$, adjusted with HCl to pH 6.8) by a Psycotron homogenizer (Microtec Co. LTD, Chiba, Japan) at 30000 rpm for 1 min. Further steps were carried out as described earlier [14]. Briefly, the samples were centrifuged and the soluble proteins in the supernatant precipitated with 95 % acetone. The lyophilized dry protein powder was subjected to separation by centrifugal precipitation chromatography or spCCC.

2.4 Solvent systems and sample preparation

2.4.1 Centrifugal precipitation chromatography

Previously, we optimized the separation conditions for carotenoid cleavage-like enzymes from tea leaves using an acetone Tris-buffer gradient [15]. A similar solvent system and gradient has been successfully applied in this study for the isolation of carotenoid cleavage-like enzymes from algae. The buffer consisted of 50 mM Tris-HCl, 125 mM KCl, 5 mM $MgCl_2$, adjusted with HCl to pH 6.8. The sample solution was prepared by dissolving 150 mg of the crude extract in 1 mL elution buffer. Prior to injection, the sample was filtered through a 0.45 µm membrane filter (Durapore 0.45 µm, Millipore, Bedford, MA, USA).

2.4.2 spCCC

We developed an aqueous two phase solvent system of PEG 1000 and mono- and dibasic potassium phosphate. The solvent system was prepared by dissolving 25 % PEG 1000 and 6.25 % mono-basic and 6.25 % di-basic potassium phosphate in 625 g HPLC water. The solutions were transferred into a separatory funnel and after equilibration to room temperature divided into upper and lower phase. The pH of the upper phase (PEG 1000 rich) was 6.9 and of the lower phase 6.7. Sample solutions were prepared as follows: 500 mg of the crude enzyme extract was dissolved in 0.5 mL upper and lower phase. Prior to injection the undisclosed constituents were pelleted by centrifugation at 12000*g* for 10 min.

2.5 Separation procedure

2.5.1 Centrifugal precipitation chromatography

The upper and lower channels of the seal-free mini-centrifuge were filled with acetone. After injection of 1 mL of the sample into the upper channel, this protein channel was eluted from the inner to the outer terminal with Tris–HCl buffer at a flow rate of 0.05 mL min^{-1}. The separation disk was centrifuged at 2000 rpm. The proteins were precipitated over 60 min with acetone, before starting the elution by increasing the buffer concentration to 100 % over a period of 300 min, and kept at 100 % buffer for another 60 min. The flow rate inside the solvent channel was 1 mL min^{-1}. Fractions were collected in intervals of 20 min at the sample channel output, evaporated to dryness, and dissolved in 0.8 mL water.

2.5.2 spCCC

The spiral column was first filled with stationary phase (lower phase) of the saturated solvent system. Then the apparatus was rotated counter clockwise at 800 rpm while the mobile (upper) phase was pumped into the internal tail terminal (top) of the column (L-i-T elution mode) at a flow rate of 1 mL min^{-1}. Fractions were collected at the outer terminal in intervals of 3 min and evaporated to dryness prior to further analysis.

2.6 Analysis of fractions

2.6.1 Enzyme assay and analysis of enzymatic reaction products

The carotenoid cleavage ability of the fractions was determined using a spectro-photometric assay as described previously [16]. β-Carotene, as one of the major endogenous carotenoids, and β-apo-8'-carotenal were used as substrates. The initial concentrations of these were 5.8 μmol L^{-1}.

The presence of β-ionone, the putative reaction product derived from the cleavage of β-carotene, was confirmed after solid phase micro-extraction (SPME) by gas chromatography mass spectrometry (GC-MS). A SPME fiber coated with 100 μm polymethylsiloxane (Supelco, Bellefonte, PA) was introduced into a headspace vial containing 1 ml enzymatic reaction mixture and 0.5 ml saturated sodium chloride solution and incubated for 1 h at 35°C. The reaction products were analyzed by GS-MS using a capillary Suplecowax column (GL Sciences Inc., Japan, 30 m, 0.25 mm ID, 0.25μm film thickness) using the following temperature program: initial temperature 50°C, maintained for 3 min, ramped to 190°C at 5°C/min, ramped to 240 ◦C at 40°C/min, and held for 3 min. The mass scan range was set to *m/z* 50-300 and the electric potential to 1.0 kV. Helium was used as carrier gas at a flow rate of 1.7 mL min^{-1}.

2.6.2. Determination of proteins by SDS-PAGE

Prior separation the samples were concentrated and desalted by centrifugal filtration (Microsep 10 kDa, PALL). To remove the PEG spCCC fractions were dialyzed (Spectra/Por MWCO 12,000-14,000, Spectrum) against Milli-Q water for 48 h. The proteins were separated on a SDS-PAGE (12.5 % acryl amide) and detected after Silver or Coomassie Brilliant Blue staining. The molecular masses were calculated based on the *RF* values of the Dual Colour Precision Plus Protein pre-stained standards (BioRad).

3 Results and discussion

3.1 Isolation of carotenoid cleavage-like enzymes by centrifugal precipitation chromatography

A bio-activity based approach is used here for the identification of carotenoid cleavage-like enzymes in macro algae for the first time. A protein extract was subjected to centrifugal precipitation chromatography that produced fractions for bio-activity determination. The abilities of the obtained fractions to cleave β-carotene are shown in Fig. 1. Higher decreases in β-carotene concentrations imply higher enzymatic activities. The enzymatic activity is peaking at fraction 9, at an estimated acetone concentration of 50 %. This calculated acetone concentration is lower than in our previous study in tea, whereas the target protein eluted at a calculated acetone concentration of slightly below 90% [15]. To confirm that the fractions (8-11) contained our target protein, we analyzed the enzymatic reaction products after head space sampling (SPME) by GC-MS and compared it to the amount of β-ionone resulting from chemical degradation processes in fractions (2-5). Carotenoids are unstable compounds which easily isomerize in the presence of light or heat and/or oxidize in the presence of oxygen or other oxidizing compounds. A higher concentration of the putative enzymatic reaction product β-ionone was obtained in fractions (8-11) (Fig. 2), which is consistent with the result obtained spectrophotometrically (Fig. 1). Previously, carotenoid cleavage-like enzymes of molecular masses of 69 kDa and 71 kDa have been isolated from tea leaves and nectarines, respectively [15, 18]. After silver-staining we obtained two major proteins of 72 and 65 kDa (I, II Fig. 3A). Both proteins also have a similar molecular weight to known recombinant CCD1 and CCD4 enzymes, which are involved in the formation of C_{13}-apocarotenoids; among them CCD1 enzymes from *Vitis vinifera* (61 kDa), *Petunia hybrida* (61 kDa), *Rosa damascena* (62 kDa*), Osmanthus fragrans* (65 kDa) or CCD4 enzymes from *Malus domestica* (62 kDa), *Rosa damascena* (64 kDa), and *Osmanthus fragrans* (67 kDa) [19-23].

3.2 Isolation of carotenoid cleavage-like enzymes by spCCC

For separation of proteins, several aqueous two phase solvent systems have been proven to be suitable for spCCC. PEG-dextran or PEG ATPS have been applied for the separation of standard proteins previously [7, 11, 12]. For the separation of myoglobin and lysozyme parameters have been optimized for ATPS consisting of PEG 1000 and dibasic potassium phosphate in spCCC [11, 12]. The partitioning of the seaweed extract was tested in solvent systems containing mono- and dibasic potassium phosphates and PEG 1000 or PEG 3350. Sufficient distribution was obtained in the solvent

system containing 25 % PEG 1000 and 6.25 % mono-basic and 6.25 % di-basic potassium phosphate. To maintain the enzymatic activity during the separation we were interested in an aqueous solvent system, where both phases have a pH of approximately 6.8. In our study the upper phase had a pH of 6.9 and the lower phase 6.7. The instrumental design of spCCC and the volume of the separation column allow higher sample loads compared to centrifugal precipitation chromatography. In this study, 500 mg crude protein extract was fractionated in a single run of 120 min. The fractions containing the target enzyme were located by activity screening. In this study we only focused on the fractions with highest activities 34 to 38 (Fig. 4). Fractions comprising lower carotenoid cleavage ability, such as (18 – 20) were not further analyzed. The proteins in fractions 34 and 35 were analyzed by SDS-PAGE. As control we used fractions with lower carotenoid degradation ability (fractions 2, 3, and 4). After dialysis two proteins of 72 and 64 kDa were obtained (Fig. 3B) in fractions 34 and 35, which equal the molecular sizes of the target proteins obtained after centrifugal precipitation chromatography (Fig. 3A). Higher amounts of target proteins were obtained from spCCC. The proteins could be detected after CBB-staining, which allows the detection in µg scale compared to silver staining methods, which detect proteins in ng levels. This amount would increase more if faster and more efficient methods for the removal of the PEG could be developed; this is a current drawback of PEG ATPS.

To determine whether our enzymes have the functionality of CCD1 and CCD4 enzymes, we used β-carotene and β-apo-8'-carotenal as substrates. Enzymes of both families can cleave carotenoids and apocarotenoids for example at 9,10 or 9',10' double bonds [18-23]. Our target fractions could utilize β-carotene as well as β-apo-8'-carotenal to produce β-ionone (Fig. 5). The molecular size of the proteins and substrate utilization suggest that our fractions contain a CCD1- and/or CCD4-like enzyme. Further studies are needed to identify which protein is responsible for the cleavage or if both proteins are active carotenoid cleavage-like enzymes.

4 Concluding remarks

This study demonstrates the potential of centrifugal precipitation chromatography and spCCC for the isolation of active bio-molecules, such as enzymes from complex biological extracts. Both

instrumentations can use solvent systems which can highly preserve enzymatic activities and can be applied as alternative purification steps in protein purification protocols. SpCCC in addition has significant capacity to isolate high quantities of enzymes.

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Figure captions

Figure 1

Relative enzymatic activities and calculated acetone concentrations of centrifugal precipitation chromatography fractions. Higher decreases in β-carotene concentrations imply higher enzymatic activities. The proteins were precipitated over 60 min with acetone, before starting the elution by changing the Tris–HCl buffer concentration to 100 % over a period of 300 min, and kept at 100 % buffer for another 60 min. The flow rate inside the solvent channel was set to 1 mL min^{-1}. The sample channel was eluted from the inner to the outer terminal pumping Tris–HCl buffer at a flow rate of 0.05 mL min^{-1} through the sample channel. Fractions were collected in intervals of 60 min at the sample channel output. The rotation speed of the separation disc was set to 1000 rpm.

Figure 2

(A) GC-MS chromatograms of β-ionone formed in the reaction mixtures of fractions 2-5 (chemical degradation) and 8-11 (chemical and enzymatic degradation). The reaction products were analyzed after SPME headspace sampling by GS-MS using a capillary Suplecowax column (GL Sciences Inc., Japan, 30 m, 0.25 mm ID, 0.25 lm film thickness) and the following temperature program: initial temperature 50°C, maintained for 3 min, ramped to 190°C at 5°C min^{-1}, ramped to 240 ◦C at 40°C min^{-1}, and held for 3 min. The electric potential was set to 1.0 kV and the helium flow rate to 1.7 mL min^{-1}. (B) Mass spectra of β-ionone detected in fractions 8-11.

Figure 3

SDS-PAGES of (A) (1) centrifugal precipitation chromatography fractions 8-11 (**I** 72 kDa, **II** 65 kDa, and (M) marker stained with Silver Staining Kit III (Wako) and (B) (M) marker, (1') spCCC fractions 2-4 , and (2') spCCC fractions 34-35 (**I'** 72 kDa, **II'** 64 kDa) stained with Bio-Safe Coomassie Blue G-250 Stain (BioRad).

Figure 4

Relative enzymatic activities of spCCC fractions. The separation was carried out in the solvent system of 25 % PEG 1000 and 6.25 % mono-basic and 6.25 % di-basic potassium phosphate. The apparatus was rotated counter clockwise at 800 rpm while the mobile (upper) phase is pumped into the internal tail terminal of the column (L-i-T elution mode) at a flow rate of 1 mL min^{-1}. Fractions were collected at the outer terminal in intervals of 3 min.

Figure 5

GC-MS chromatograms (*m/z* 177; abundant molecular ion in the mass spectra of β-ionone as shown in Fig. 2) of (A) authentic β-ionone, (B) β-ionone derived from the cleavage of β-carotene (C) β–ionone derived from the cleavage of β-apo-8'-carotenal

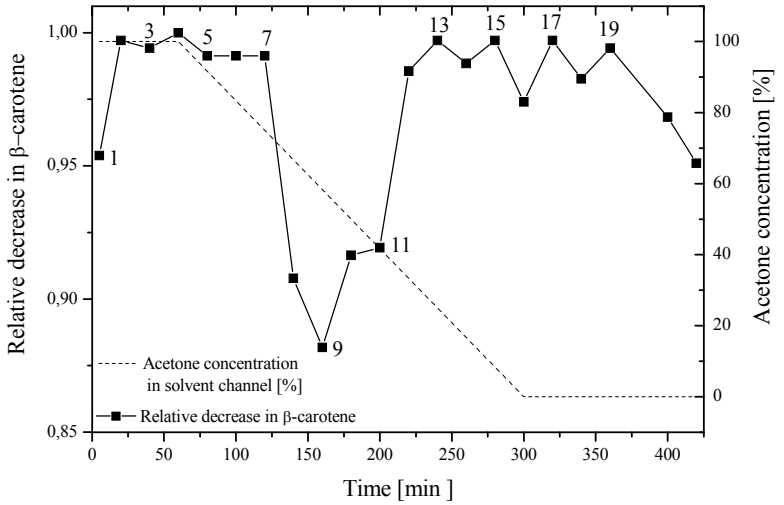


Figure 1: Baldermann *et al.*

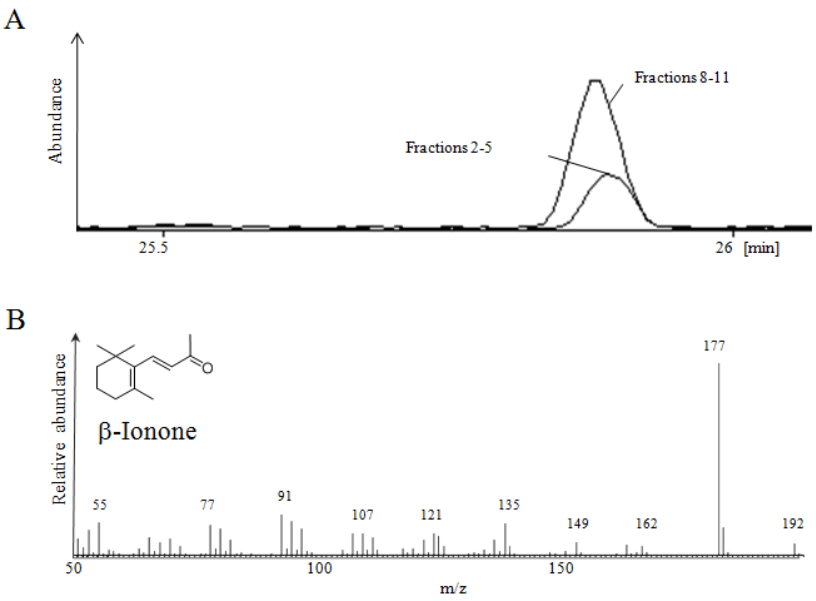
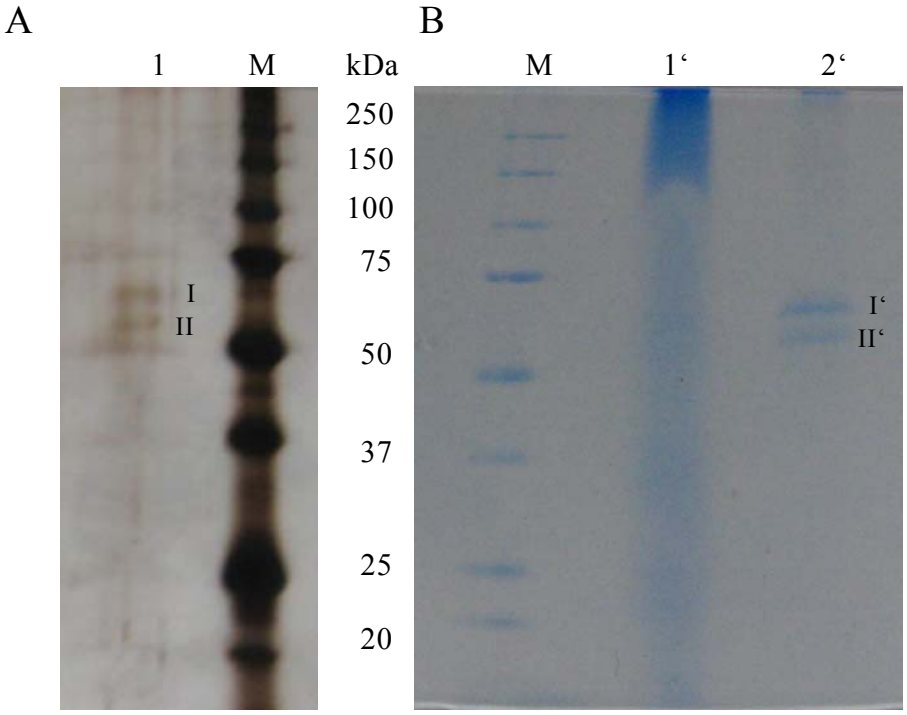


Figure 2: Baldermann *et al.*



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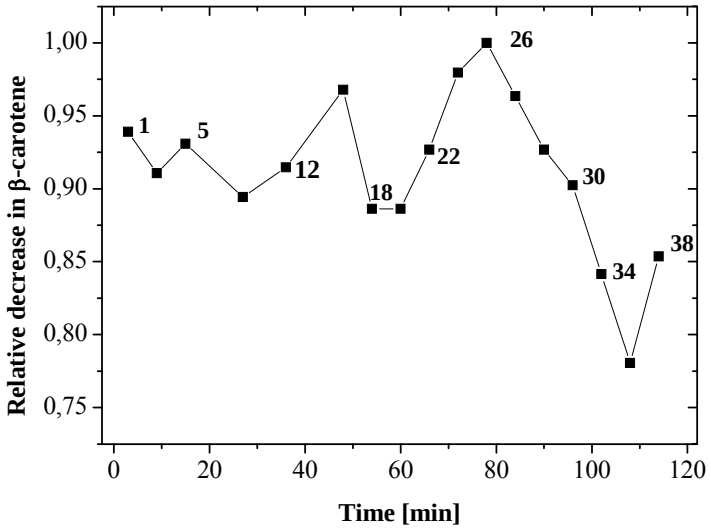

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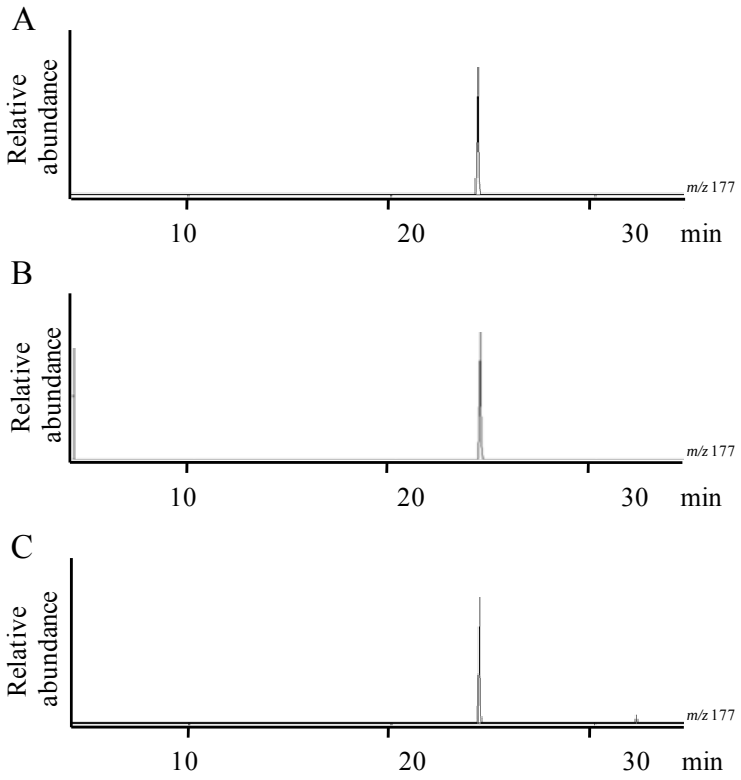

Figure 5: Baldermann *et al.*