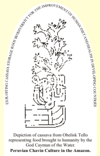
Chromoplast associated proteins from color storage root diversity in cassava (*Manihot esculenta* Crantz)[1].



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ABSTRACT

Accumulation of β-carotene in underground storage organ might result from a variety of regulatory mechanism, including steps in the syntheses pathway and its end product sequestration in the chromoplast. Such mechanisms may be especially important in non-green tissues performing massive carotenoid biosynthesis, such as in some clones of cassava (*Manihot esculenta* Crantz) storage roots. Recently, in our laboratory, specific carotene form accumulation in the storage root of cassava has been observed to parallel the high protein content in the storage root due to the occurrence of a larger protein population difference in chromoplast-associated proteins when the color cassava was compared with white one. Surprisingly, the protein content correlated with high amount of a particular form of carotenoid presence. These observations are further reported in the present communication. Results so far indicated that total soluble protein content in the storage root of color cassava reach more than 40% the amount of protein commonly reported for white cassava. Two dimensional gel electrophoresis analyses indicated that the number of proteins of a chromoplast-enriched suspension is about three times in color cassava in comparison to the identical tissue fraction extract from the white cassava. Protein size exclusion chromatography of the chromoplast-enriched suspension displayed three groups of chromoplast-associated proteins coming in association with three distinct patterns of carotenoid accumulated in traditional clone of cassava. These complex sets of protein groups were further characterized and partially purified in analytic RP-HPLC to forward our understand of the mechanism of carotenoid sequestering protein and the high protein content in the storage root of traditional cassava from Amazon.

INTRODUCTION

The edible storage root (SR) of cassava (*Manihot esculenta* Crantz) has been considered in the past, solely as a source of starch. However, to improve cassava for carotenoid pigmentation is an open and interesting area of research considering the beneficial effects of this compound class acting as antioxidants and provitamin A and given the prevalence of vitamin A deficiency diseases in Brasil (www.saude.gov.br, Flores 1998, Marinho et al 1981). Genetic diversity for cassava SR color is available in several germplasm collections maintained in India (Moorthy et al. 1990), Brazil (Guimaraes e Barros 1971; Marinho et al. 1996) and Colombia (Iglesias et al 1997). Studies with an orange-pigmented SR clone indicated that two cycles of selection and recombination could improve the amount of β-carotene as the major provitamin A carotene. The inheritance of β-carotene accumulation was reported as controlled by the action of two genes with complete dominance of orange over white SR trait (Iglesias et al. 1997). These reports underscore the high potential of cassava for development towards as an improved staple food combining macronutrients (starch) with provitamin A as a micronutrient. However, the few SR pigmented clones obtained so far may not represent promising genetic materials to develop provitamin A rich cassava because of their relatively low levels of β-carotene (ranging from 0.75 to 4.69mg/100g FW). More recently, expeditions to the center of origin and domestication of cassava in the Brazilian Amazon has been able to collected additional genetic diversity for the SR carotenoid content trait, including clones with the color varying from white, intense yellow, cream and pink (Carvalho et al. 2000a, Carvalho et al. 2000b).

Carotenoid biosynthesis represents a molecularly well-characterized biochemical pathway in plants. Over the last ten years rapid progress has been made in the cloning of genes encoding enzymes of the carotenoid pathway using several different approaches and strategies such as color complementation in *E. coli* and the molecular identification of mutants (Cunningham and Grantt, 1998). To date, practically all structural genes involved in plant carotenogenesis are known; only -ring hydroxylation remains to be elucidated. While there is a growing wealth of data available showing how differential expression of these structural genes affects carotenoid content or pattern, no regulatory genes have been identified so far.

Accumulation of β-carotene in the cassava storage root might be the result of a variety of regulatory mechanisms (Cunningham and Grantt, 1998). Besides transcriptional regulation, other mechanisms may be important as well, such as product sequestration. Such mechanisms may be especially important in non-green tissues performing massive carotenoid biosynthesis, such as in cassava roots. In chromoplasts, carotenoids are a synthesized membrane-bound product with the final lipophilic product being delivered into the lipid-bilayer. The massive carotenoid deposition will ultimately lead to deleterious alteration of the physico-chemical properties of membrane, which need to be avoided. In fact, chromoplasts develop sites of carotenoid deposition in the stroma. These are lipid globules (plastoglobules) and/or proteins organizing fibrillar supramolecular structures containing carotenoid and lipids. The formation of carotenoid crystals is another principle of carotenoid sequestration as well as the proliferation of plastid membranes (Camara et al., 1995). Sequestration, i.e. the removal of end products from their site of formation in membranes may create chemical disequilibria driving a pathway towards completion. In the unicellular algae *Dunaliella* this seems to be the case, where the inhibition of lipid accumulation in the form of lipid globules resulted in the incapability of the system to over accumulate carotenoid. To identify and to provide such cellular sinks for carotenoid may add to the arsenal of technology currently available to modify this biosynthetic pathway in transgene approaches such as in rice grains (Ye et al., 2000). Therefore the chromoplast-associated protein turns out to be an important issue in improving protein content in cassava storage root.

In this document we are reporting results towards the exploitation of the natural genetic diversity of SR color in cassava to improve knowledge on the carotenoid accumulation in cassava. Our approaches includes the description of the occurrence of carotenoid types in cassava storage root, their differential accumulation during root development, the identification of chromoplast-associated proteins, and finally the isolation of candidate genes controlling the differential accumulation of carotenoid using the diversity of storage root color in cassava clones.

MATERIALS AND METHODS

Plant material: Cassava clones from our GENEBANK composed of traditional cassava clones collected in Amazon displaying a diverse range of SR colors were used in the present study (Figure 1a). A set of these clones, cultivated in field plots at Embrapa Genetic Resources and Biotechnology, were classified in five color classes and used for quantification of carotenoid (previously reported) and aqueous soluble proteins to address questions related to clone diversity and storage root development. The most representative individual from each class of pigmented clone was processed separately for chromoplast isolation and characterization of chromoplast associated proteins.

Tissue preparation: Cylinders of storage roots with 30-40 cm long and 4-6 cm diameter were manually sliced or dissected in individual tissue layers (Figure 1b), immediately frozen in liquid nitrogen, freeze-dried and stored in -80°C until use for protein quantification. For the chromoplast-associated proteins studies, fresh intact storage roots were peeled off and freshly processed immediately after harvest.

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Soluble protein extraction and quantification: One gram of dried tissue was added with 20ml of acetone (100%), incubated in water bath at 50°C for 30', followed by the addition of 2ml of extraction buffer (SDS 5%, Glycerol 10%, Tris 80mM pH 6.8, and DTT 25mM), cooled at room temperature, incubated at 20°C for more than 1h to obtain a protein precipitate free of pigments. After centrifugation (30000rpm/4°C/20') the supernatant was drained, the pellet dried by blowing N2 air and suspended with 5ml of extraction buffer (EB). The re-suspended solution was centrifuged again (30000rpm/4°C/10') to collect 1ml aliquot of the supernatant that was stored in 80°C until use. Aliquots of 100µl were treated with DOC (0.15%) and TCA (12%) to precipitate aqueous soluble proteins (ASP). After centrifugation (13000rpm/4°C/20') the pellet was suspended in 100µl of EB, and 50µl used for tissue protein estimation by the BCA method using the Micro Kit of PIERCE according to the manufacturer.

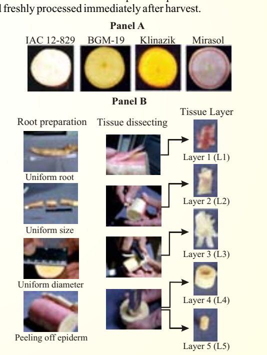

Figure 1 - Color diversity and storage root tissue sampling system. **Panel A** shows the close up of the cross section of the major color diversity observed in the field trip. **Panel B** shows the tissue sampling system of the tissue system I (Layer 1 L1), II

Chromoplast enriched suspension isolation: Intensely colored layers of fresh storage roots from four contrasting color clones (IAC 12-829, Klinazik, BMG19 and Mirasol) were separated, sliced, and treated with homogenate buffer (Tris-HCl 100mM, pH 8.2, EDTA 8mM, KCl 10mM, $MgCl_2$ 2mM, sucrose 400mM, and PMSF 1mM) for 2 hours in a cold room and ground with pulses with a household blender to obtain a paste that was filter through a three layers of cheesecloth. The filtrate was centrifuged (400rpm/4°C/20') and the supernatant collected for a new centrifugation (20000rpm/4°C/40'). The resulting pellet, enriched with chromoplast, was suspended in 100ml of homogenate buffer (HB), washed once with HB followed by centrifugation (20000rpm/4°C/40'). The pellet was collected, re-suspended in 100ml of suspension buffer (Tris-HCl 100mM pH6.8, 250mM NaCl), sonicated, centrifuged (20000rpm/4°C/45') and the supernatant aliquots of 50ml was split and used for protein population analysis by 2-DE gel (fraction **ASCrAP** for Aqueous Soluble Chromoplast Proteins) and purification by gel filtration chromatography (fraction **SSCrAP** for Solvent Soluble Chromoplast Proteins).

Aqueous Soluble Chromoplast Proteins (ASCrAP) characterization by 2-DE gel: An aliquot of 1mL from the ASCrAP fraction was precipitated with DOC (0.15%) and TCA (12%) and centrifuged (13000rpm/4°C/40'). The pellet was washed twice with 85% cold acetone and suspended in 360µL rehydrating IPG buffer (8M Urea, 2% CHAPS, 2% IPG buffer-pH 3-10, 25mM DTT, and bromophenol blue). IPG dry strips (pH 3-10 linear) of 18 cm long was rehydrated with proteins sample and focused according to Gorg et al. (2000). pI standard (2-DE standard molecular marker - BioRad) was also treated IPG buffer and focused in separating gel. The second dimension protein separation used an ISODALT gel system from Pharmacia according to the manufacturer in a 13% SDS-PAGE. Molecular weight (BenchMark Protein Ladder - Invitrogen) marker for SDS-PAGE gel was also included in the second dimention gel. Two-dimensional gel images were generated using a high resolution (1024x1024 pixel) Dual Scan (AGFA T2000) and transferred to a high capacity computer. Images were analyzed with the GELLAB II+ software (Scanalytics). Images were processed to select region of interest, background correction, image contrast equivalence among gels, molecular weight and pI standard calibration, and relative OD reads within and across gels of a particular experiment using white cassava (IAC 12-829) as reference gel. Experiment was repeated twice and high quality gels with reproducibility were used to identify spots in the sample and estimation of pI, MW and %OD reads.

Gel filtration of non-denatured Chromoplast Associated Proteins: An aliquot of 5ml from the extraction fraction **SSCrAP** was loaded to a column with a resin-bed (Sepharose CL 6B-200) of 1m long per 1.5 cm internal diameter previously equilibrated with suspension buffer (SB). Fractions of 1ml were collected with a flow rate of 1.5ml/min for OD readings at 280nm and 461nm in spectrophotometer (SHIMADZU model S2000). Fractions from the three observed peaks were pooled separately, concentrated in an AMICON cell with a 3000Da membrane cut off and stored at 80°C until further analyzed in RP-HPLC. Each extraction was repeated three times.

Reverse phase HPLC: Pooled peak fractions from the gel filtration chromatography were dried in a speed-vac and the pellet re-suspended in 500µl acetonitrile with 2% TFA to dissociate protein-pigment complex. After evaporation the acetonitrile, the proteins were suspended in 500µl of mqH_20 with 2% TFA and loaded to a analytical Hamilton column (214TP1010, 1.0cmX25cm, C-18) in a HPLC system (SHIMADZU, LC-10AD, VP) equipped with a UV/VIS detector (SHIMADZU, SPD-10AV, VP). Proteins were separated with linear gradient of the solvent system A-2%TFA/mqH2O to B-Acetonitrile/2%TFA in the mobile phase under a flow rate of 1ml/min. Peaks were collected, dried in a peed-vac, and stored in refrigerator until being used for purity quality verification in a MALDI-Tof.

RESULTS AND DISCUSSIONS

Protein content in clones of cassava with diverse color: Acetone powder aqueous soluble protein content in cassava storage root showed in Figure 2 indicates a somewhat unexpected trend of close association between higher protein content and pigmentation of storage root (Figure 2 Panel A). It is observed that the protein content in pigmented SR can reach values superior to 40% of the protein content in the white SR clone. This higher amount of proteins in color clones may be related the amount of chromoplast-associated protein needed to stabilize and accumulate the carotenes during its deposition in the chromoplast (Vishnevetsky, et al 1999). Therefore, if this protein content in storage root of pigmented cassava clones holds truth as the causal mechanism of the high protein content, it may be manipulated to increase protein content in cassava storage root. Figure 2b shows the pattern of distribution of aqueous soluble protein in different tissue layers of the storage root. Protein content varies among tissue system, being tissue system I (L1) and II (L2) with the highest protein content. In tissue system III there is no general trend in the variation of protein content in the layers (L3, L4, L5), indicating that the age effect observed in the accumulation of carotenoid (previously reported) may not directly related to the protein content at this level of observation.

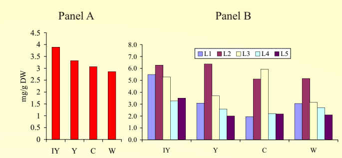

Figure 2 - Protein content and distribution in relation to color and tissue layers. **Panel A** Mean values of aqueous soluble protein content in four different classes of cassava storage root color. **Panel B** Distribution of

Two dimensional gel analysis of chromoplast associated proteins: The soluble protein associated with chromoplast from storage root of cassava was analyzed by 2-DE gel. Figure 3 shows the 2-DE gel map for the aqueous protein extract from the clones studied and display the spot number identification for the unique proteins for each type of cassava. Table 1 summarizes the analysis of the number of paired protein spots across clones of distinct color and color intensity. The number of protein spots is always bigger in color cassava than in white cassava. The overall characterization of the soluble chromoplast-associated proteins is shown in Figure 4.

Table 1 - Pairing comparison of chromoplast-associated proteins from four classes of storage root color. Protein spots were separated and visualized in the 2-DE gel analysis.

Cassava clone	Number of spots observed		Pairing comparison			
	Total	Unique	IAC	BGM	KLI	MIR
IAC	50	24	-	6/84	21/138	9/142
BGM	84	56	6/50	-	19/138	16/142
KLI	138	84	21/50	19/84	-	30/142
MIR	142	98	9/50	16/84	30/138	-

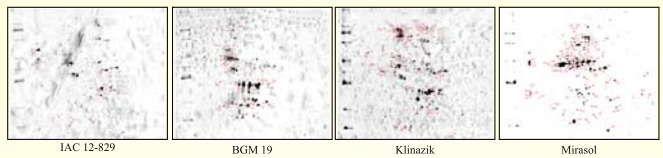

Figure 3 - Profile of 2-DE gel electrophoresis of chromoplast-associated proteins from pigmented storage root of cassava.

Chromoplast associated protein characterization by gel filtration: Non-denatured protein-pigment complex suspension was used to purify chromoplast-associated protein in size exclusion chromatography in complementing to the technical limitations of work with highly hydrophobic proteins in the 2-DE gel system. Figure 5 shows the size exclusion chromatography elution pattern of the non-denature protein-pigment complex detected by the Abs_{461} (for the pigment) and Abs_{280} (for the protein). Three fraction peaks of proteins-pigment complex were observed in all clones, indicating the different chromoplast associated proteins from the diversity of storage root color phenotype. While the clone BGM19 (Figure 5b), which accumulate solely β-carotene showed a single peak (fraction 28-30) of protein-pigment complex, the clone Klinazik (Figure 5c), which accumulate β-carotene and lutein, showed two peaks (fraction 28-30 and fractions 85-88). The clone Mirasol (Figure 5d), which accumulate β-carotene and lutein as well as lycopene and others pigment types, showed the same corresponding fraction peaks and some other minor peaks. A small amount of protein-pigment complex was also observed in the early peak (fractions 28-30) of the white cassava (Figure 5a), which does not match with the observation that this cultivar did not show detectable amount of carotenoid (previous results). The early peak elute within the void volume of the column as determined with blue dextran (MW 2000kD). This peak with a non-denatured protein-pigment complex around 2000kD is similar to the one observed for β-carotene protein-complex in carrot

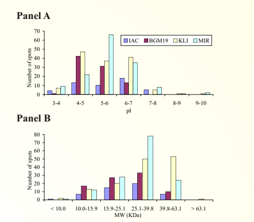

Figure 4 - Chromoplast-associated proteins distribution based on its pI and MW. **Panel A** Protein distribute based on pI. **Panel B** Protein distribution based on Molecular Weight. Proteins spots were separated, visualized, identified and characterized in 2-DE gel electrophoresis.

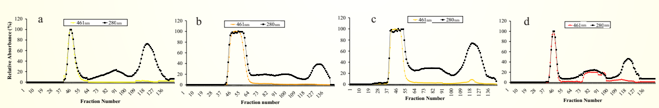

Figure 5 - Gel filtration chromatography (Sepharose CL 6B-200) of non denature protein-pigment complex from chromoplast enriched extract from four different clones of cassava. Protein and pigments were monitored at 280 and 461nm respectively. (a) - fraction for variety IAC 12-829 (white cassava), b) - fraction profile for clone BGM19 (yellow cassava), (c) - fraction profile for clone Klinazik (Intense yellow cassava) (d) - fraction profile for clone Mirasol (Pink cassava).

Partial purification of chromoplast associated protein by RP-HPLC: Figure 6 shows the protein profile of the corresponding peaks I, II, and III from the gel filtration chromatography (Figure 5). At this level of purification the protein-pigment complex was denatured with acetonitrile to remove the pigments and the hydrophobic proteins separated in reverse phase high performance liquid chromatography (RP-HPLC) in a C18 analytic column. The number of pigment-associated protein species varies among clones, the elution time of protein species varies among clones and peaks from the previous purification step. The clone Mirasol (pink cassava) showed the largest number of protein peaks in the RP-HPLC profile in all three peaks from the previous purification step (Panel B.1, B.2, B.3), while the variety IAC 12-829 (white cassava) showed the least complex protein profile for each corresponding peak (Panel B.1, B.2, B.3).

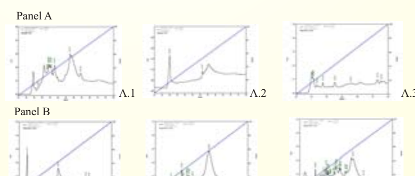

Figure 6 - Sample comparison of dissociated protein-pigment complex between white and pink cassava. RP-HPLC profiles of dissociated protein-pigment complex corresponding to the three peaks in the gel filtration chromatography from Figure 3. Proteins were separated in a linear hydrophobic gradient of acetonitrile (0-100%) with a C18 analytic column and detected at 216nm. **Panel A** profile corresponding peak I (A.1), II (A.2), and III (A.3) for variety IAC 12-829 (White cassava). **Panel B** - profile corresponding peak I (B.1), II (B.2), and III (B.3) for clone Mirasol (pink cassava).

CONCLUDING REMARKS

The results presented here, opens new avenues in cassava research. The protein requirement for the accumulation of carotenoid could be explored as a mechanism to enrich total protein content of the cassava storage root. A more conclusive analysis is under way to identify the protein specificity to a particular pigment by taken the advantage of the diversity of the clones identified. Finally, the bioavailability and the thermoprocessing studies of the protein-pigment complex would further determine their impact in the human nutrition

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