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β-Carotene Micellarization during in Vitro Digestion and Uptake by Caco-2 Cells Is Directly Proportional to β-Carotene Content in Different Genotypes of Cassava[1,2]

Sagar K. Thakkar,[3] Bussie Maziya-Dixon,[5] Alfred G. O. Dixon,[5] and Mark L. Failla[3,4]*

[3]Interdisciplinary PhD Program in Nutrition, and [4]Department of Human Nutrition, The Ohio State University, Columbus, OH 43210; and [5]International Institute of Tropical Agriculture (IITA), P.M.B. 5320, Ibadan, Nigeria

Abstract

Cassava, a staple food in sub-Saharan Africa, does not provide adequate amounts of pro-vitamin A (VA) carotenoids and has been targeted for biofortification (i.e. selectively breeding cultivars of increased nutrient density with agroeconomically acceptable characteristics). However, the accessibility of pro-VA carotenoids for absorption in different cultivars of cassava remains unknown. Here, we used the coupled in vitro digestion/Caco-2 cell uptake model to screen the relative accessibility of β-carotene (βC) in 10 cultivars of cassava with varying concentrations of βC. After cooking (boiled for 30 min), the βC concentration in tubers from different cultivars ranged from less than detectable to 6.9 μg βC/g cassava. Samples were subjected to simulated oral, gastric, and small intestinal digestion to determine stability and micellarization of βC. All-*trans* βC, 9-*cis* βC, and 13-*cis* βC were the most abundant carotenoids in cooked cassava and recoveries after digestion exceeded 70%. Efficiency of micellarization of total βC was 30 ± 2% for various cultivars with no significant difference in isomers and linearly proportional to concentration in cooked cassava ($r = 0.87$; $P < 0.001$). Accumulation of all-*trans* βC by Caco-2 cells incubated with the diluted micelle fraction for 4 h was proportional ($R^2 = 0.99$; $P < 0.001$) to the quantity present in micelles. These results suggest that all-*trans* βC content appears to provide the key selection marker for breeding cassava to improve VA status and that the more complicated screening procedure using in vitro digestion coupled to cell uptake does not provide additional information on potential bioavailability. J. Nutr. 137: 2229–2233, 2007.

Introduction

Vitamin A (VA)[6] deficiency continues to be a global public health problem, especially in developing countries of sub-Saharan Africa and Southeast Asia where the diet is generally restricted to staple plant foods with low concentrations of pro-VA carotenoids (1). VA deficiency leads to night blindness (nyctalopia) and xeropthalmia that can progress to permanent loss of vision if the deficiency is prolonged. Inadequate VA status is also associated with impaired resistance to infection and increased mortality rates for infants, children, and pregnant and postpartum women. Traditionally, the 3 main strategies to prevent and treat this micronutrient deficiency have been dietary diversification, food fortification, and medicinal supplementation. These approaches are relatively cost effective but have failed to eradicate VA deficiency because of lack of diverse food supply; problems with distribution, especially in rural areas; and lack of compliance (2). Genetic engineering and biofortification represent alternative and sustainable strategies to improve the micronutrient density of food crop. Biofortification involves screening the germplasm for accessions rich in limiting micronutrients for cross-breeding with varieties with agronomically acceptable characteristics to generate nutrient-dense, high-yielding cultivars (3,4).

Cassava (*Manihot esculenta* Crantz) is an important food source in tropical Africa where annual consumption exceeds 80 kg per capita (5). It is estimated that 70 million people obtain >500 kcal/d (2.1 MJ/d) from cassava. However, cassava is a poor source of protein, iron, zinc, and pro-VA carotenoids and contains toxic cyanogenic glucosides. The strategy to biofortify cassava with pro-VA carotenoids assumes that the additional complement of the pigments and their bioactive metabolites will be delivered to target tissues, i.e. it will be bioavailable. The bioavailability of carotenoids depends on numerous factors, including physico-chemical properties of the carotenoid, food matrix, method of processing, presence of promoters and inhibitors of carotenoid absorption in the meal, and the nutritional status and general health of the individual (6). The relationship between pro-VA content and bioavailability in cassava is unknown.

The absorption of carotenoids, like other fat-soluble compounds in a meal, requires transfer from the food matrix to emulsified oil droplets followed by partitioning into mixed micelles during digestion in the small intestine, uptake into

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[6] Abbreviations used: βC, β-carotene; DI, deionized; VA, vitamin A.
* To whom correspondence should be addressed. E-mail: failla.3@osu.edu.

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enterocytes, and incorporation and secretion in chylomicrons. Accessibility refers to the transfer of the compound of interest from the food matrix to intestinal absorptive cells. The coupled in vitro digestion/Caco-2 cell uptake model has been proposed as a cost-effective, relatively high-throughput system for screening the accessibility of carotenoids (7) and iron (8,9) from foods, meals, and supplements. Such screening is needed to help plant breeders identify promising nutrient-dense genotypes to cross with high-yield varieties in subsequent planting seasons. In vitro digestion was recently validated as a relevant model for estimation of in vivo accessibility of carotenoids (10). The objective of this study was to determine the accessibility of β-carotene (βC) in cultivars of cassava with varying concentrations of βC using the in vitro/Caco-2 cell model. The results suggest that βC content of cultivars of cassava is an acceptable marker for the generation of varieties enriched in bioavailable βC.

Materials and Methods

Chemicals and supplies. Unless otherwise stated, all chemicals and supplies were purchased from Sigma-Aldrich and Fisher Scientific.

Cassava varieties. Yellow-fleshed cassava genotypes were grown under rain-fed conditions in 2005–2006 in a randomized complete block design with 2 replications at the research farm of the International Institute of Tropical Agriculture, Ibadan, Nigeria. Planting was done at the beginning of the rainy season (May to June). The varieties are tolerant to the major pests and disease of cassava. In addition, the varieties are high yielding. No fertilizer or herbicides were applied during the course of the experiment and hand weeding was done when necessary. At harvest, 12 mo after planting, 10 genotypes were selected based on the analysis of pro-VA carotenoid content conducted during the previous year. Three roots of different size (large, medium, and small) were selected for each genotype, washed, air-dried, packed in moist saw dust, and shipped to the US.

Preparation of cassava. Skin was peeled and tips from the distal and proximal portions (1–2 cm) were removed. Cubes (3–5 cm^2) were cut and submerged in deionized (DI) water at room temperature to remove cyanogenic glucosides. The water was changed every 2 h for 10 h. The cubes were then submerged overnight in DI water before boiling them in 10 parts of DI water for 30 min. This treatment is similar to the manner in which cassava is generally prepared for human consumption. Boiled cassava was allowed to cool at room temperature and mashed before storage in 50-mL polypropylene screw-cap tubes with a blanket of nitrogen gas at −80°C. All the steps were performed with minimal light.

In vitro digestion. Boiled cassava (3 g) was subjected to simulated digestion as described by Garrett et al. (7) with several modifications intended to better reflect physiologic conditions in the gut. The in vitro digestion consisted of simulated oral, gastric, and small intestinal phases of digestion. Because of its high starch content, the oral phase of digestion (10 min, 37°C) was included according to Oomen et al. (11), with the exception that that 3000 units α-amylase was added per gram cassava. A basal salt solution containing NaCl, KCl, and $CaCl_2$ (final concentrations 120, 5, and 6 mmol/L, respectively) was substituted for 150 mmol/L as the basal solution for simulated gastric and small intestinal digestion. KCl was added as another physiological salt besides NaCl and $CaCl_2$ was added for maximal activity of lipases. The pH of gastric digestion was adjusted to 2.5 ± 0.1 instead of 2.0 ± 0.1 and that of small intestinal digestion was adjusted to 6.5 ± 0.1 instead of 7.0 ± 0.1. Porcine pancreatic lipase (final concentration 0.2 g/L in 100 mmol/L $NaHCO_3$) in addition to pancreatin and bile extract to facilitate lipid digestion. Finally, the micelle fraction was isolated from digesta by centrifugation at 5000 × *g*; 45 min at 4°C and filtration (0.22 μm pore size) of the collected aqueous fraction, instead of the previously described high speed centrifugation (167,000 × *g*; 35 min) followed by filtration. Pilot studies showed that carotenoid concentrations in the filtered aqueous fractions using the 2 different centrifugation speeds did not differ.

Uptake of βC by Caco-2 human intestinal cells. Stock cultures of Caco-2 (HTB-37) cells were obtained from American Type Culture Collection and were maintained as previously described by our laboratory (12,13) except that DMEM containing Piperazine-1,4-bis(2-ethanesulfonic acid) (15 mmol/L, pKa = 6.8) was substituted for HEPES as a buffer in cell culture medium. Because of relatively low concentration of βC in cassava cultivars, cells were cultured in T-25 flasks for experiments. Cultures of Caco-2 at passages 31–35 were seeded in T-25 flasks at 2×10^4 cells/cm^2 and used for experiments between 12 and 14 d after reaching confluency.

Extraction of carotenoids from digesta, micelle fraction, cells, and cassava tubers. Carotenoids were extracted from digesta, micelle fraction, and Caco-2 cells as described by Garrett et al. (12). The extraction of carotenoids from cooked cassava was adopted from Kimura et al. (14). Sudan I was used as internal standard and its recovery suggested 97 ± 2.5% efficiency of extraction of carotenoids from samples (15).

HPLC analyses. Separation and quantification of carotenoids was achieved using a Waters YMC Carotenoid S-5 C_{30} reversed-phase column (4.6 mm × 250 mm; particle size, 5 μm) and HPLC system described by Chitchumroonchokchai et al. (16).

Statistical analysis of data. A minimum of 3 independent digestions and cell uptake studies were made for each test sample in an experiment and each experiment was replicated at least once to generate a minimum of 6 observations for each cultivar. All statistical analyses were performed using SPSS (version 14.0, SPSS). Data are presented as means ± SEM. Differences were considered significant at $P < 0.05$. Means were compared by ANOVA with Tukey's post hoc test. Simple linear regression analysis was performed to test the relation between the βC concentration in boiled cassava and the quantity of the carotenoids incorporated into micelles generated during simulated digestion of cassava. The relationship between the content of all-*trans* βC accumulated in Caco-2 cells when the monolayers were exposed to diluted fraction of micelles generated simulated digestion and the amount of all-*trans* βC in cooked cassava cultivars was assessed using Pearson correlation analysis.

Results

Carotenoid composition of cassava. All-*trans* βC, 9-*cis* βC, and 13-*cis* βC were identified in extracts from all tested cultivars of boiled cassava (**Fig. 1***A*). The absorption spectrum of a minor peak eluting at 15 min (peaks at 471, 442, 422, and 338 nm) suggests the presence of a *cis* isomer of zeaxanthin, although this remains to be confirmed. The total βC concentration in 10 cultivars ranged from less than the limit of detection (<0.05 pmol/g, cultivar no. 10) to 6.9 μg/g of cassava (Fig. 1*B*). All-*trans* βC was the most abundant isomer of βC present in all cultivars of boiled cassava. However, the relative amount of all-*trans* βC clustered in 2 groups (i.e. 48–55% for cultivar nos. 3, 5, 8, and 9 and 65–70% for cultivar nos. 1, 2, 4, 6, and 7). 13-*cis* βC content ranged from 20–27%, whereas 9-*cis* βC varied from 5–28%.

Digestive stability and micellarization of βC during in vitro digestion. Recoveries of the 3 isomers of βC after simulated oral, gastric, and small intestinal phases of digestion exceeded 70% and were not significantly different ($P > 0.05$). The ratio of *cis* and *trans* isomers was not altered during digestion. The amount of βC incorporated into micelles ranged from 0.3 to 2.3 μg/g of boiled cassava in various cultivars. The efficiency

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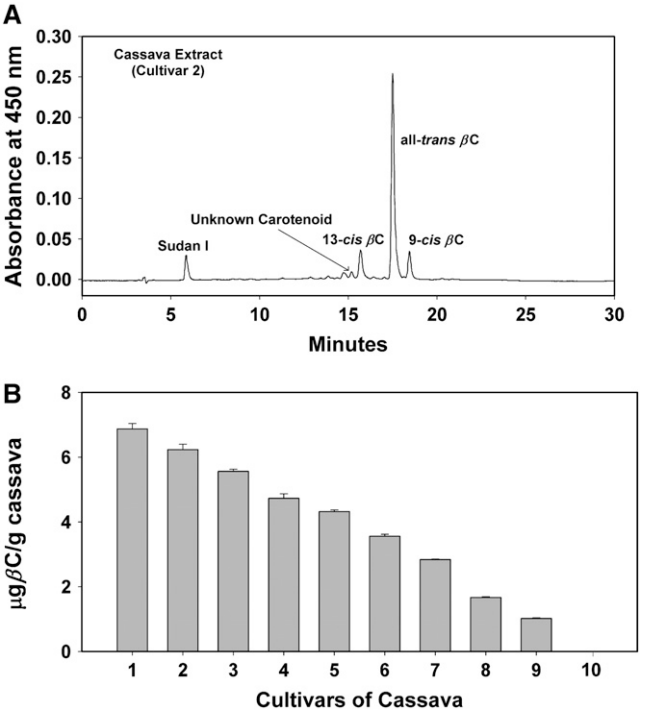

FIGURE 1 Carotenoid composition (*A*) and βC concentrations (*B*) in cultivars of cassava. In *A*, a representative chromatogram of carotenoids extracted from boiled cassava cultivar no. 2 is shown. βC isomers were identified by comparison of elution profiles and absorption spectra. Sudan I was added to cassava as a recovery standard before extraction. In *B*, values are means ± SEM for 3 independent extractions from each cultivar.

of micellarization of βC following small intestinal digestion was 30 ± 2% and the relative extent of micellarization for all-*trans* βC, 9-*cis* βC, and 13-*cis* βC did not differ. The total βC concentration in cassava cultivars and the quantity of the carotenoid incorporated into micelles were correlated ($r = 0.87$; $P < 0.001$) (**Fig. 2**).

Uptake of micellar carotenoids by Caco-2 cells. The micelle fraction generated during simulated digestion was diluted (1:4) with DMEM supplemented with 1% nonessential amino acids and L-glutamine (2 mmol/L) to examine cellular accumulation of βC. Total amount and the isomeric profile of micellar βC were stable during incubation for 4 h in 95% air:5% CO_2 atmosphere in a humidified cell culture incubator at 37°C. Monolayers of Caco-2 cells maintained normal morphological appearance during 4-h incubation with diluted (25%, v:v) micelle fraction from digested cassava. A pilot study with cultivar number 2 showed that Caco-2 cells preferentially accumulated ($P > 0.05$) all-*trans* βC (10.0 ± 1.1%) and 9-*cis* βC (9.1 ± 0.9%) from the medium compared to 13-*cis* βC (5.4 ± 0.5%). The experiment was repeated by incubating monolayers in T-25 flasks with diluted micellar fractions generated during digestion of boiled cassava containing >2 μg all-*trans* βC per gram cassava (i.e. cultivar nos. 1–5). Cellular accumulation of all-*trans* βC ranged from 1.4 to 7.5 ng/mg cell protein and was linearly proportional ($R^2 = 0.99$; $P < 0.001$) to the quantity present in micelles generated during simulated digestion (**Fig. 3**). All-*trans* βC in cells represented 10.8–11.5% of that in medium. The amount of all-*trans* βC accumulated by Caco-2 cells after 4 h incubation was correlated ($R^2 = 0.92$; $P < 0.001$) with the amount of this isomer in medium. The concentrations of 9-*cis* and 13-*cis* βC in cells were too low to accurately quantify uptake from medium containing micelles generated during digestion of genotypes 1–5.

Discussion

The continued prevalence of micronutrient deficiency in many developing regions of the world necessitates the development of new varieties of staple food crops that are enriched in limiting nutrients (3). In addition to increased nutrient density, it is essential that at least some of the additional nutrient is absorbed and delivered to target tissues for efficacy. The absorption of carotenoids requires their release from the food matrix and incorporation into mixed micelles during small intestinal digestion, uptake by small intestinal mucosal cells, and efflux into lymph via chylomicrons. The primary objective of this study was to examine the relationship between βC content in cassava and its accessibility by examining the efficiency of βC micellarization during in vitro digestion. The assumption is that the results obtained from the in vitro study are indicative of micellarization in vivo. Borel et al. (10) recently reported that accessibility as determined by in vitro digestion was correlated with data derived by sampling the small intestinal luminal contents from

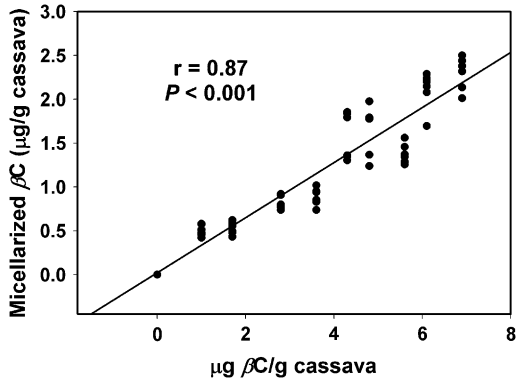

FIGURE 2 Regression analysis of total βC concentration in cassava (cultivars 1–10) and the quantity of the carotenoid incorporated into micelles generated during simulated digestion of cassava. Data are means ± SEM for 6 replicates for each cultivar.

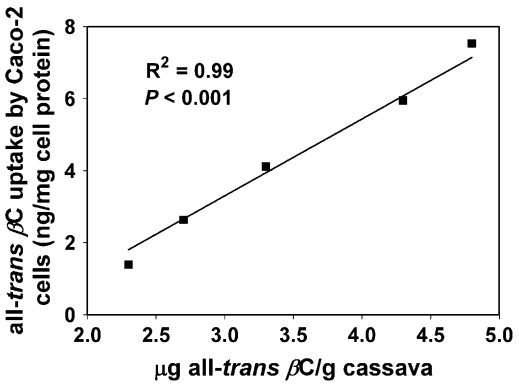

FIGURE 3 Pearson correlation analysis of all-*trans* βC content in cassava (cultivars 1–5) and the quantity of all-*trans* βC accumulated by Caco-2 cells when monolayers were exposed to diluted micelle fraction generated during simulated digestion for 4 h. Data are means ± SEM for 4 replicate cultures treated with diluted micelle fraction.

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human subjects fed carotenoid-rich vegetables and bioavailability data from published human studies. Here, we examined 10 cultivars of cassava with increasing amounts of total βC for analysis. Our results clearly demonstrate: *1*) similar recoveries of all-*trans*, 9-*cis*, and 13-*cis* βC after simulated oral, gastric, and small intestinal digestion; *2*) the amount of βC partitioned into micelles during digestion was linearly proportional to concentration in cultivars of cooked cassava; and *3*) accumulation of all-*trans* βC by differentiated monolayers of Caco-2 cells was proportional to the concentration in micelles. Collectively, the results suggest that content of all-*trans* βC in the germplasm of cassava represents a useful marker for selecting cultivars for crossing with varieties possessing appropriate agroeconomic characteristics to create βC-rich, high-producing varieties of cassava without the apparent need for routine in vitro and animal studies to assess relative accessibility and bioavailability, respectively. It is noteworthy that liver VA reserves in Mongolian gerbils are correlated with increasing content of dietary pro-VA carotenoids in biofortified maize (17).

The observation that recovery of βC after simulated oral, gastric, and small intestinal digestion of cassava exceeded 70% agrees with previous reports that carotenoids in other fruits and vegetables generally are stable during in vitro digestion (12,16,18,19) and during passage through the upper gastrointestinal tract of human subjects (20,21). The isomeric profile of βC also was similar before and after digestion of cooked cassava as observed for βC supplements (18). In contrast to the in vitro model, limited isomerization of all-*trans* βC has been observed during in vivo digestion. The relative amount of 13-*cis* βC was greater in duodenal lumen than in gastric lumen of subjects fed pureed carrots and tomato (20). Also, the ratio of 9- and 13-*cis* to all-*trans* βC in the residual contents in stomach of gerbils increased compared to that in oil after oral administration (22).

Micellarization of βC during digestion of cassava was independent of genotype, isomeric structure, and total content of the carotenoid. Approximately one-third of the βC was transferred from boiled cassava to mixed micelles during the small intestinal phase of simulated digestion. The relative degree of βC micellarization during digestion of cassava was greater than that reported during in vitro digestion of other vegetables and fruits, including carrot, spinach, tomato, and pumpkin (13,16,19,23). Moreover, the efficient micellarization of βC during digestion of cassava occurred in the absence of exogenous fat, a promoter of carotenoid bioavailability, and its conversion to VA (24). Direct comparison of relative extent of micellarization may be misleading, because the actual quantity of βC incorporated into micelles during small intestinal digestion of cassava was much less than during the digestion of the indicated foods containing higher amounts of this carotenoid. Numerous factors including subcellular location and physical state of the carotenoids affect the transfer of these pigments from the food matrix to micelles (6,10,25,26).

Uptake of βC from micelles generated during simulated digestion of the 5 cultivars of cassava with the highest concentration of the carotenoid was examined to confirm accessibility. Caco-2 cells accumulated all-*trans* βC in a concentration-dependent manner, whereas *cis* isomers of βC were not detected. Assuming equivalent uptake efficiencies of uptake for *cis* and all-*trans* βC (10–11% of medium), cell content of *cis* isomers was expected to be 0.1–0.25 pmol/mg cell protein. Because our limit of detection was 0.05 pmol βC (signal-to-noise ratio for peak height = 5), the absence of *cis* βC suggests that the all-*trans* isomer is more efficiently transported than *cis* isomers. Indeed, uptake of all-*trans* βC from Tween 40 micelles by Caco-2 cells was 4- to 7-fold >9-*cis*- and 13-*cis* βC (27). The recent demonstration that uptake of carotenoids by small intestinal cells is protein mediated (28,29) offers a likely explanation for such isomer specificity. It also is possible that *cis* βC was isomerized to all-*trans* βC in micelles or after transport into Caco-2 cells. However, we previously reported that the isomeric profile of βC and xanthophylls is stable in both micelles in culture medium and after accumulation by Caco-2 cells maintained under standard culture conditions (16,23,30). During et al. (27) also demonstrated that uptake of all-*trans* βC was directly proportional to medium βC at concentrations ranging from 0.1 to 5 μmol/L but curvilinear between 5 and 20 μmol/L. Thus, the high correlation between micellar and cell concentrations of all-*trans* βC in our study was expected, because medium contained only 0.02–0.04 μmol/L all-*trans* βC.

Cooking disrupts plant cell walls and organelle membranes, facilitating greater access of digestive enzymes to substrates and release of carotenoids for incorporation into mixed micelles (31,32). Processing of plant foods also induces isomerization of carotenoids, thus increasing the levels of *cis* isomers. For example, baking is associated with isomerization and degradation of all-*trans* βC in sweet potatoes (33). Although *cis* isomers of βC also are precursors of VA, their retinol activity equivalence is only one-half that of all-*trans* βC (34). The cis βC content of the 10 genotypes of cooked cassava analyzed in this study was relatively high, ranging from 30 to 52% of the total βC content. Cassava was cooked (boiled) and frozen as soon as the tubers arrived in our laboratory. Because we did not analyze samples of raw tuber, the contributions of genotype, style of cooking, and the genotype by cooking interaction on the observed ratio of *cis*:all-*trans* βC requires future investigation. Nevertheless, high content of all-*trans* βC appears to represent the primary factor for selecting cultivars of cassava for crossing with agroeconomically fit varieties.

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