



Preparation of Cassava Starch Nanoparticles and their Application as a Carrier System for Anthocyanin and Carotene Delivery

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Abstract

Cassava is a tropical tuber crop useful in the production of high quality starch economically. One interesting application of starch is in the preparation of starch nanocrystals and nanoparticles by acid hydrolysis. These nanoparticles possess a reactive surface covered with hydroxyl groups, providing the possibility of extensive chemical modification. Biodegradable nanoparticles have been recently suggested as controlled release constructs to mitigate the side effects caused by nonspecific action of cytotoxic drugs commonly employed in chemotherapy. In this connection, starch nanoparticles currently receive much attention because of the abundance and easy availability of starch, its low cost, renewability, biocompatibility, biodegradability, and nontoxicity. There are several research reports supporting the capacity of these nanoparticles as excellent candidates for implant materials and drug carriers. Anthocyanin, a flavanoid extracted from the leaves of sweet potato, is a very good natural chemo preventive agent that allows suppression, retardation or inversion of carcinogenesis. The chemo preventive effect of carotene is also well established. In the present work, the preparation of starch nanoparticles from cassava starch, their characterization using transmission electron microscopy (TEM) and atomic force microscopy (AFM) and their interaction with anthocyanin and carotene are attempted. The evidence for loading was provided by attenuated total reflectance (ATR) spectroscopy. These plant pigments with proven chemo preventive properties after incorporation into starch nanoparticles could possibly be used in pharmaceutical applications since these nanoparticles possess excellent drug carrier properties.

Key words: Cassava, starch nano particles, carotene, anthocyanin

Introduction

Great progress has been achieved in the development of starch as a renewable carbohydrate polymer, procurable at low cost from a great variety of crops. The low cost of this biopolymer and its biodegradability, are the major reasons leading to the growing interest in the nonfood usage of starch-based products for applications in which synthetic polymers have traditionally been the materials of choice. One interesting application of this biopolymer is in the preparation of starch nanocrystals and nanoparticles by acid hydrolysis (Dufresne et al., 1996; Putaux et al., 2003; Angellier et al., 2004; Habibi and

Dufresne, 2008). The starch nanoparticles, for their properties qualitatively different from those of native starch granules, could be utilized in new applications. Polysaccharide nanoparticles possess a reactive surface covered with hydroxyl groups, providing the possibility of extensive chemical modification (Dufresne, 2010). Angellier et al., (2004) reported that their work consisted of optimizing the preparation of nanocrystals from waxy maize starch granules using sulfuric acid hydrolysis. The use of starch nanoparticles currently receives much attention because of the abundant availability of starch, its low cost, renewability, biocompatibility,

biodegradability, and nontoxicity. The latter properties make these nanoparticles excellent candidates for implant materials and drug carriers (Brigger et al., 2002).

Tropical tuber crops contain starch as the major component and thus act as an important source of starch. Cassava (*Manihot esculenta* Crantz) and to a small extent, sweet potato (*Ipomoea batatas* Lam.) are used for starch extraction in many regions of the world. Studies on different starches at ICAR-Central Tuber Crops Research Institute (ICAR-CTCRI, Thiruvananthapuram, India) and elsewhere have brought to light the wide diversity in the starch characteristics of tuber crops (Moorthy, 2002). Extraction of starch from cassava is simple and the isolated starch is pure white in colour and relatively free from other chemical impurities. The total amylose content in cassava starch has been reported to range from 13.6-23.8% (Rickard et al., 1991).

Even though anthocyanins possess excellent antioxidant and anticancer properties, their bioavailability is poor (He et al., 2006). Attempts have been made through encapsulation in polymeric micelles, liposomes, polymeric nanoparticles, lipid-based nanoparticles, and hydrogels to increase its aqueous solubility and bioavailability (Ma et al., 2008; Bisht et al., 2007). The present work aims at the preparation of starch nanoparticles from cassava starch and to characterize them using TEM and AFM. Since starch nanoparticles are excellent drug carriers, loading them with natural pigments like anthocyanin and carotene which have widely accepted antioxidant and chemopreventive action, forms the objective of the study. Since the potential of starch nanoparticles as drug carriers has been extensively studied (Brigger et al., 2002), the present work was undertaken to prepare and characterize starch nanoparticles from cassava and to develop it as a carrier system for curcumin which could possibly be used in pharmaceutical applications.

Materials and Methods

Fresh cassava tubers immediately after harvesting were used for starch preparation. β -Carotene was purchased from Sigma Aldrich. All other solvents used were of analytical grade. Hydrolysis of the starch was performed on the platform of New Brunswick orbital shaker (GMI, USA) kept at room temperature. For centrifugation of the hydrolyzed starch, a Hermle Z-36-6 refrigerated

centrifuge (Hermle Labortechnik, Germany) was used. TEM images were observed using a Jeol 1001 Transmission Electron Microscope (Jeol Ltd., Japan) at 80 KV accelerating voltage. AFM was performed using a NT-MDT Digital Instrument (NT-MDT, Russia) operating in the tapping mode region. ATR measurements were performed on IR-Prestige spectrophotometer (Horiba Scientific, Japan) with ATR facility.

Preparation of aqueous suspension of cassava starch nanoparticles

Starch was extracted from fresh cassava tubers as described elsewhere (Badenhuizen, 1965) and was dried in an air oven to make it moisture free, and submitted to acid hydrolysis as reported by Angellier et al. (2004). A given weight (7.0 g) of moisture free native cassava starch powder was mixed with 50 ml of 3.16 M sulphuric acid solution in a 100 ml Erlenmeyer flask. The reaction mixture was stirred using a shaker kept at $30 \pm 2^\circ\text{C}$ and continuously stirred for five days at 100 rpm speed with an orbital shaking action. After 5 days, the suspension was washed by successive centrifugations for 10 minutes at 10,000 rpm, with distilled water until neutrality. The suspension was then submitted to a mechanical treatment with homogenizer Ultra Turrax for 2 minutes at 13,000 rpm to disperse aggregates to obtain a "stable" suspension. The resulting suspension was stabilized by sulphate groups present at the surface of nanoparticles gained from the H_2SO_4 treatment (Angellier et al. 2005). To avoid bacterial growth during storage, a few drops of chloroform were added to the suspension that was kept in refrigerator.

The experiment was repeated with different weights of native starch, 5.0g, 7.0g and 10.0g. When 5.0g was taken as the initial weight of native starch, practically no residue was obtained after 5 days hydrolysis, but solid unhydrolysed residue was obtained when the initial weight of the starch taken was 7.0g and 10.0g.

Characterization of starch nanoparticles

Transmission Electron Microscopy (TEM)

After a brief sonication, a drop of dilute starch nanoparticle suspension was deposited onto a glow discharged formvar-coated microscopy grid. After 1 min, the liquid in excess was blotted with filter paper and the remaining film was allowed to dry. Once positioned into a specimen holder, the grid was transferred into the microscope, and observed at room temperature.

All specimens were observed using a Jeol 1001 Transmission Electron Microscope (Jeol Ltd., Japan) at 80 KV accelerating voltage. Micrograph was recorded.

Atomic Force Microscopy (AFM)

AFM was performed using a NT-MDT Digital Instrument (NT-MDT, Russia) operating in the tapping mode region. Micro-fabricated silicon Cantilever tips (MPP-1100-10) with a resonance frequency of 299 KHz and a spring constant of 20–80 nm⁻¹ were used. The scan rate varied from 0.5 to 1.5 Hz. AFM analysis was done offline. Starch nanoparticles for the imaging were prepared by drop casting the suspension on freshly cleaned mica at the required concentration and examined under ambient conditions. In order to rule out the possibility of any artifacts, blank experiments were carried out with neat solvents (without test material) on mica. Scanning at various planes showed the neat surface of mica without the morphology of any objects.

Aggregation of starch nanoparticle suspension

The cassava starch nanoparticle suspension when refrigerated after adding a few drops of chloroform was stable for a long period. These particles had a tendency to unite with each other and to form aggregates. The particle size was analysed immediately after preparation and after two months of storage and found that aggregation of particles occurs on long standing. One interesting behavior of this suspension was that, when acetone was added in drops to the starch nanoparticle suspension after filtration through a Millex GP (Millipore) filter having a pore size of 0.22 µm (220 nm), the nanoparticles got aggregated as shown in Fig. 3. The test at the right side of the figure clearly showed the aggregated nanoparticles. By centrifuging the mixture obtained after adding sufficient acetone for obtaining maximum aggregation, at 10,000 rpm for 10 minutes, the aggregated starch nanoparticles were recovered. This was then washed using methanol and vacuum dried. This solid easily became a gel, as soon as it came in to contact with atmospheric air. This residue was used to record the solid fluorescence spectrum and FTIR spectrum.

Preparation of anthocyanin rich extract (ARE)

Coloured anthocyanic leaves from the sweet potato genotype S-purple were collected from the experimental field of sweet potato germplasm collection of ICAR-Central Tuber Crops Research Institute and anthocyanin

pigment was extracted using methanol containing 5% trifluoro acetic acid as described by Rajeswari *et al.*, (2010). The extract was concentrated in a flash evaporator and purified by Amberlite XAD-7 column chromatography. This was again concentrated by flash evaporation to get ARE and kept stored at -18°C.

Loading of carotene and anthocyanin into starch nanoparticles

Starch after hydrolysis as mentioned above, was diluted and filtered carefully through a Millipore filter having a pore size of 220 nm and the filtrate was collected. This filtrate was immediately treated with carotene and ARE in acetone (0.005 molar) in the ratio 3:1 in separate screw capped flasks and mixed well and kept for 8 hours. To separate the modified starch particles after the reaction, sufficient amount of acetone was added to aggregate the starch particles and centrifuged. The residue was then washed with methanol to remove adhered pigments till the filtrate became colorless, and was refrigerated.

Characterization of starch nanoparticles after loading with pigments using ATR spectra

ATR measurements were performed on an IR-Prestige spectrophotometer (Horiba Scientific, Japan) with ATR facility. A small volume of the pigment incorporated starch nanoparticles in methanol was added to the groove and placed in the sample holder directly and spectrum was recorded in wave number range from 400–4000cm⁻¹. The pure pigments were separately dissolved in methanol and their ATR spectra were also recorded.

Results and Discussion

TEM images

TEM micrograph of starch nanoparticles is shown in Fig. 1. This micrograph clearly showed that the starch nanoparticles obtained after 5 days of sulphuric acid hydrolysis had the shape of aggregates of spherical nanoparticles with majority of them observed in the size range of 50–100 nanometers. In the figure there are white dots surrounded by a gray halo corresponding to individualized or aggregates of several starch nanoparticles.

AFM analysis

AFM images of starch nanoparticles are shown in Fig. 2. From the figure it is clear that majority of particles were having a particle size of around 100 nm. Results

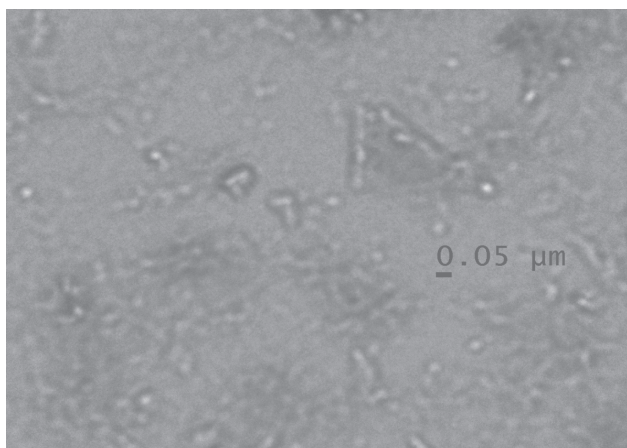


Fig.1. TEM of starch nanoparticles obtained after hydrolysis of cassava starch granules

from TEM and AFM imaging clearly showed that the particles produced were spherical with particle size in the nano scale; the structural integrity of the nanoparticles was confirmed. In contrast to the shape of nano particles produced from waxy maize, which were obtained as platelets of nanocrystals (Angellier et al., 2004), these particles were spherical. The shape and particle size of granules depends strongly on its botanic origin (Corre et al., 2010). The large surface area, inherent to the small size of nanoparticles, guarantees a large surface activity and a high grafting per unit mass of particles (Angellier et al., 2005). Szymonska et al. (2008) prepared cassava and potato starch nanoparticles by grinding the starch-ethanol suspensions in a vibration mill. Mixture of the processed granules was separated by sedimentation into polysaccharide fractions and the fractions after 36 hours of sedimentation (about 15% of the mixture) were

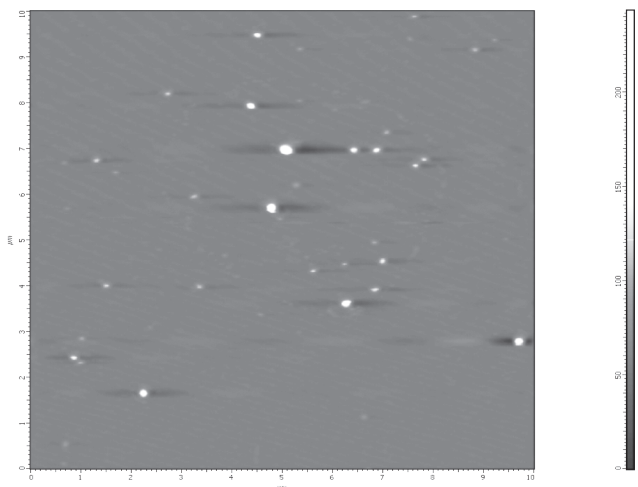


Fig.2. AFM image of cassava starch nanoparticles

collected. They determined physicochemical properties of starch nanoparticles and found that these particles had a high aqueous solubility and swelling power compared to native starch which indicated that the particles fit the amylopectin type short branched species. Disadvantages of this method were that the stabilization of the particles by the sulphate groups was not there, and that the mechanical processing of starch caused severe damages to the granules.

Aggregation of starch nanoparticles

The aggregation of starch nanoparticles on adding acetone is shown in Fig. 3. This behavior of starch nanosuspension on adding acetone can be explained on the basis of the agglomeration of the nanoparticles. Since these particles were not showing this behavior towards methanol, it can be assumed that, polarity of the solvent also plays an important role. The starch nanoparticles are believed to aggregate as a result of hydrogen bond interactions due to the surface hydroxyl groups. Blocking these interactions by relatively large molecular weight molecules obviously improves the individualization of the nanoparticles (Angellier et al., 2004). Angellier et al. (2005), estimated the hydroxyl group content present at the surface of freeze-dried starch nanoparticles to be approximately 14% of the total amount available, i.e., in 1 g of freeze-dried starch nanocrystals, only 0.0025 mol of hydroxyl groups were reactive. This indicated that only the polar hydroxyl groups sitting at the surface of starch nanoparticles are

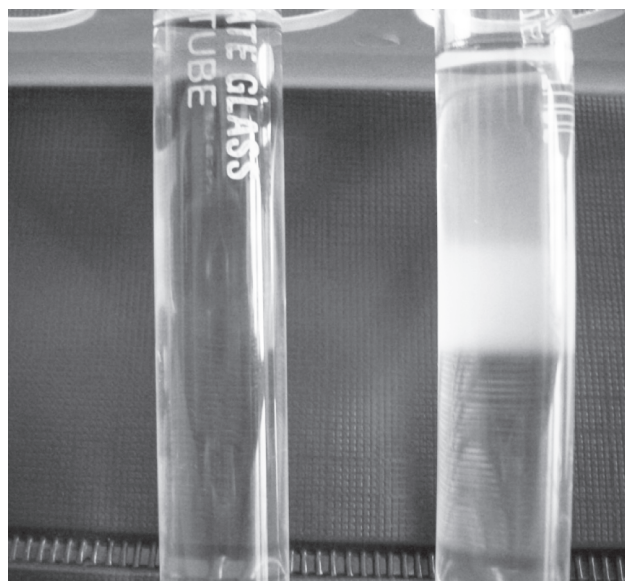


Fig.3. Aggregation of starch nanoparticles (left) after adding acetone (right)

available for chemical modification, and others remain intact within the particle, so the morphology of the starch nanoparticle skeleton can be kept unchanged, even after interaction.

ATR spectra analysis

ATR spectra of starch nanoparticles loaded with ARE

The ATR spectra of ARE, SNP loaded with ARE and starch nanoparticles (SNP) are shown in Fig. 4. The ATR spectrum of starch nanoparticles showed a very broad and strong band at 3564 cm^{-1} , suggesting presence of hydroxyl groups in them. This was lowered and shifted to a higher wave number, 3680 cm^{-1} , suggesting that the hydroxyl groups in the curcumin loaded starch nanoparticles were drastically decreased. The substitution of hydroxyl groups in a modified SNP by other functional groups from the ARE decreased the total number of hydroxyl group so that the strong hydrogen bonds were weakened. Similarly the peak at 800 cm^{-1} which was present in the ATR spectrum of ARE was lowered and reduced to a very small peak near 800 cm^{-1} .

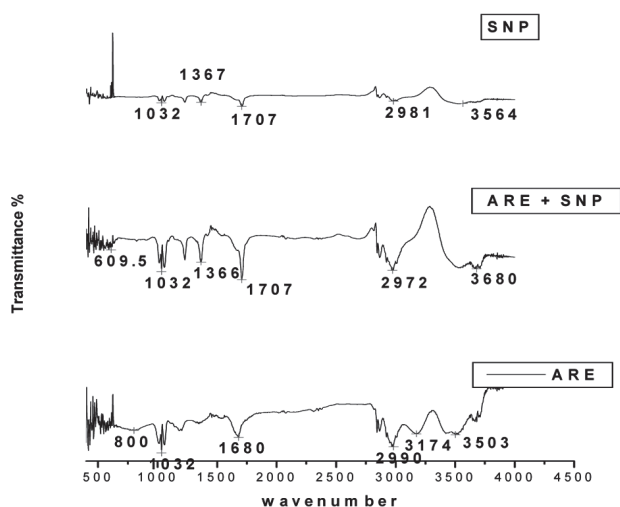


Fig.4. ATR spectra of ARE, starch nanoparticles (SNP) and SNP loaded with ARE

ATR spectra of starch nanoparticles loaded with carotene

The spectral data of SNP and carotene loaded SNP are given in Fig. 5. The spectral details suggested that the very broad and strong band at 3564 cm^{-1} , characteristic of the hydroxyl groups in SNP was narrowed and shifted to a higher wave number, 3680 cm^{-1} , suggesting that the hydroxyl groups in the loaded starch nanoparticles were

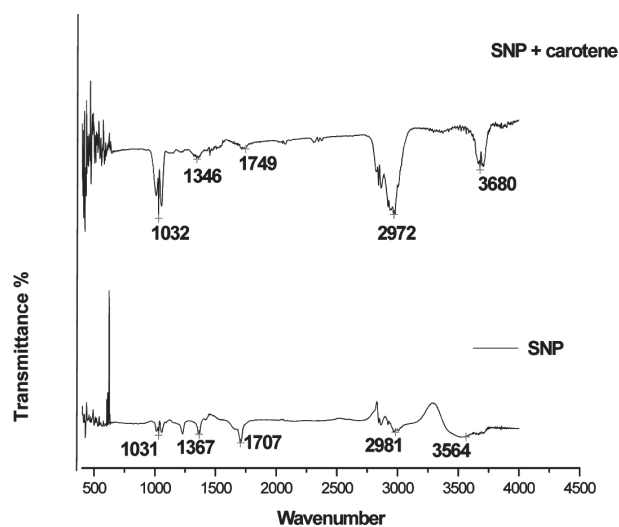


Fig.5. ATR spectra of SNP and carotene loaded SNP

substantially decreased as a result of loading with carotene. In addition to this, peaks at 1319 cm^{-1} , 1722 cm^{-1} and 2077 cm^{-1} present in the ATR spectrum of carotene (Fig.6) were shifted to 1346 cm^{-1} , 1749 cm^{-1} and 2075 cm^{-1} , respectively as a result of loading into starch nanoparticles.

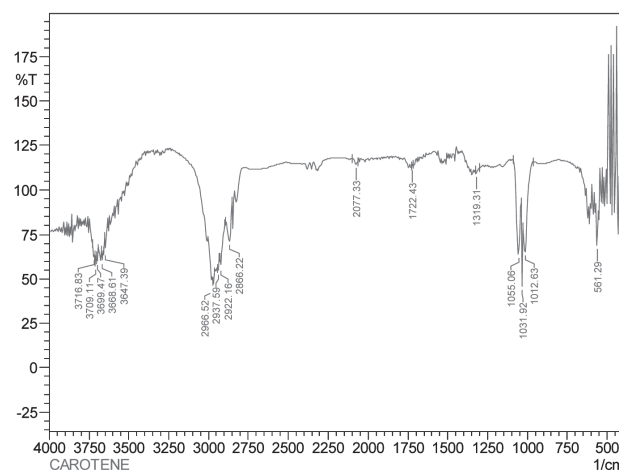


Fig.6. ATR spectra of carotene

Conclusion

Cassava starch nano particles were successfully produced by subjecting cassava starch to sulphuric acid hydrolysis. TEM and AFM images indicated the particle size in the nano scale; with majority of particles having a size around 100 nanometers and these were spherical in shape. These starch nanoparticles were stable for several months, when refrigerated. The main advantage of this method over the

mechanical grinding procedure (Szymonska et al., 2008) was that the nanoparticles attained inherent stability due to the presence of sulphate groups at their surface gained from the H₂SO₄ treatment (Angellier et al., 2005). Carotene and anthocyanin loading on the starch nanoparticles was confirmed by ATR spectroscopy. These plant pigments with proven chemo preventive properties after incorporation into starch nanoparticles could possibly be used in pharmaceutical applications since these nanoparticles possess excellent drug carrier properties (Brigger et al., 2002; Dufresne, 2010).

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