Encapsulation of genistein in amylose complexes

R. Cohen, Y. Orlova, M. Kovalev, Y. Ungar, U. Lesmes and E. Shimoni*
Faculty of Biotechnology & Food Engineering, Technion - Israel Institute of
Technology, Haifa 32000, Israel, eshimoni@tx.technion.ac.il

Introduction

Isoflavones are natural bioactives associated with a variety of beneficial health effects including reducing the risk of cardiovascular disease, lowering the rate of some cancers and improving bone health among other claims (Kano et al., 2006). One of the most abundant aglycone form of isoflavones found in soybean is genistein, 4',5,7-trihydroxyisoflavone (Crupi et al., 2007). Genistein bioavailability is limited due to its poor water solubility which limits its efficacy when administered orally (Motlekar et al., 2006). Moreover, its bitter taste is also a drawback (Huang et al., 1982). Therefore, a novel delivery system might help improve its performance.

Amylose, a fraction of starch, is known for its ability to form molecular complexes with a variety of ligands (Conde-Petit et al., 2006). These complexes are composed of left handed helices known as V amylose. The exact location of the guest molecules in V-amylose varies (inside the helix cavity, between the helices or in both sites) and so does the number of glucose residues per turn which can be six (V6), seven (V7) or eight (V8) (Biais et al., 2006). This form has recently gained attention as a possible delivery system for various bioactives, however, scarce data exists on the complexation of water-insoluble aromatic bioactives. Thus, the challenge was to induce amylose complexation with genistein, study their structure, their stability in different pH levels and temperatures, and test their functionality in simulated stomach and intestinal conditions.

Materials and methods:

Materials: Potato amylose (99% purity) and pancreatin (amylase, 41 USP; from porcine pancreas) were obtained from Sigma Chemical Co. (St. Louis, MO). Genistein was purchased from LC Labs (Woburn, MA). All other reagents were of analytical grade.

Methods: *Preparation of complexes*: Complexation was carried out via acidification of an alkali solution (Cohen et al., 2008). Amylose was dissolved in 0.1N KOH solution at 90 °C. Genistein solution was prepared separately at 30 °C (1.5 mg/ml). The solutions were mixed at 30 °C and the mixture was precipitated by adjusting the pH to 4.7 (±0.5) using 2% H_3PO_4. Complexes were either analyzed in their suspension form or separated by centrifugation (20,000g, 25 min, 4 °C), and lyophilization. *Characterization of complexes*: complexes' crystalline nature was studied by measuring the powder **X-ray diffraction** (XRD) (Philips PW 3020 diffractometer). Samples collected from suspensions were used for particle size analysis by **light scattering** techniques [LS 230 Coulter Counter particle size analyzer (Coulter Corporation, FL, USA) and dynamic light scattering (DLS) (Santa Barbara, CA, USA)], or deposited on mica for **atomic force microscopy** (AFM) scanning on a JPK Nano Wizard II AFM (JPK Instrument Inc., Germany) operating at contact mode using a cantilever with Rc<10nm. *Functional characterization:* Genistein content in the complexes and the amounts released in all experiments were quantified by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) (Griffith et al., 2001). *(A) Impact of different pHs and temperature on stability:* 15mg of the complexes were incubated in phosphate buffer (0.2M) or citrate buffer (0.1M) (ph ranges of 3 to 8) for 24 at 30 °C. Temperature stability was evaluated by complexes' incubation at 30, 50 and 80 °C for 24h. *(B) Stability in simulated stomach conditions.* Complexes (15 mg) were incubated with 1 ml of HCL pH = 2, for 2 h at 37 °C. *(C) Release in simulated gut conditions:* 15mg of complexes were incubated in either 1 ml of pancreatic amylase (35 unit/ml) at 37 °C for 24 h or pure PBS as control.

Results and discussion

*Characterization of the complexes***:** XRD examination of the complexes showed distinct reflections as shown in **Figure 1a**. This diffraction pattern differs from that of amylose-genistein physical mixture (**Figure 1b**), from amylose, processed in a similar way but without genistein (**Figure 1c**) and from that of pure genistein (**Figure 1d**). this diffractogram indicates a new structure formation in the presence of genistein, which fits previously reported structures of V6 polymorph III complexes (Buleon et al., 1990; Biais et al., 2006). In this polymorph helices are spaced, which suggests genistein might be located between the helices.
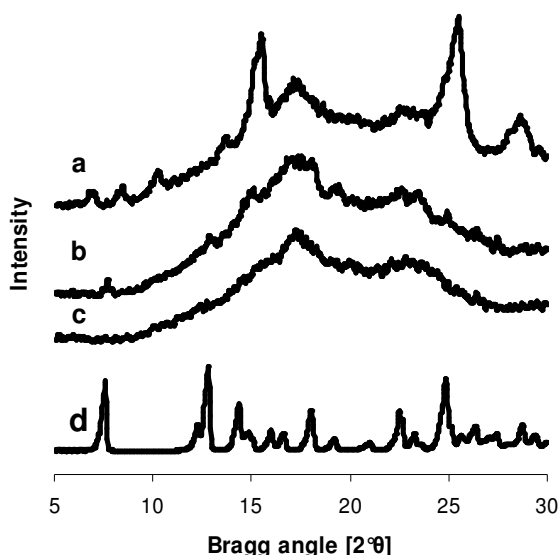

Figure 1: X-ray diffraction patterns of genistein complexes with amylose (a) genistein-amylose physical mixture (b) amylose without genistein (c) and pure genistein (d).
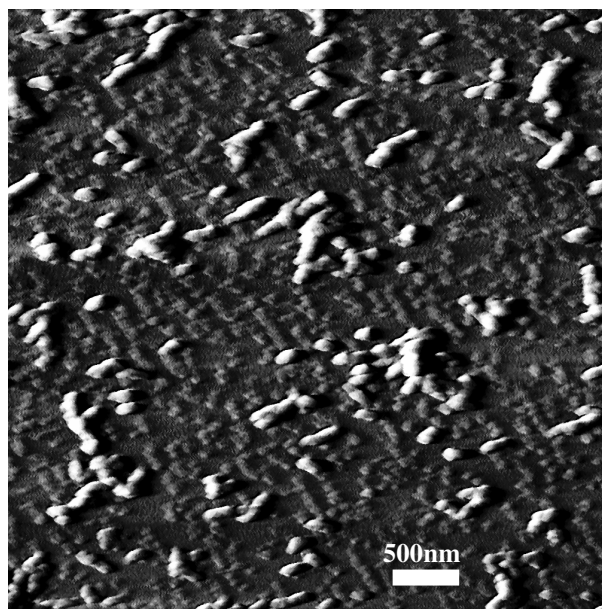
Figure 2: AFM image 4.5μm X 4.5μm of V-type amylose complexes with genistein. The sample was diluted 50 times.

Particle size analysis showed particles are about 78 ± 66 μm. The amylose particle size without the guest molecule was about two orders smaller than with genistein (148 ± 101 nm). This suggests that in an acidic environment, genistein induces some interactions between amylose molecules, which then tend to aggregate. This notion is supported by the AFM images which reveal that the particles formed are actually aggregates of much smaller particles. AFM measurements show that the mean particle size of genistein amylose complexes is actually 200 nm ± 90 nm, smaller than what was measured by light scattering (**Figure 2**). Furthermore, the effect of sample dilution on the proper dispersion of the particles and the minimization of aggregation also indicates that complexes tend to aggregate.

Release of genistein: (A) Impact of different pH and temperature on the release of genistein: Protecting the guest molecule from heat and low pH levels during processing, storage and consumption is imperative for the stability of a delivery system. It is clearly seen in **Figure 3** that the complexes were stable under different pHs, with less than 10 % of the encapsulated genistein being released. The relatively higher percentage of genistein release at pH as high as 8, can be explained by the alkali conditions, which may result in partial complex dissolution. In addition, spontaneous release of genistein rises with temperature, starting from less than 7 % at 30 $^{\circ}$C to about 25 % at 80 $^{\circ}$C (**Figure 4**).

(B) Stability in simulated stomach conditions. One of the reasons for genistein complexation is to prevent its early release in the gastrointestine. Therefore, the stability of the complex was measured in

simulated stomach conditions (HCL, 1 M, 2 h, 37 $^{\circ}$C). The release of genistein in these conditions was less than 15 % of the total amount of genistein in the complexes (**Figure 5**).

(C) Enzymatic digestion. The ultimate goal of this delivery system is to deliver the bioactive to specific sites in the digestive tract, to protect them in the acidic environment of the stomach, and eventually to release them in the gastro intestine due to enzymatic hydrolysis of the amylose. The amount of genistein released was compared to the amount released spontaneously in PBS (24 h at 37 °C). In the amylose complexes, 20.2 ± 8.1 % of total genistein were released by enzymatic digestion, whereas in PBS only 9.9 ± 4.6 % of genistein were released (**Figure 5**). These results demonstrate that although the genistein molecules are not located inside the helices, they are tightly physically entrapped between them. This location provides the genistein molecules protection from the acidic environment of the stomach while ensuring larger release under enzymatic digestion.
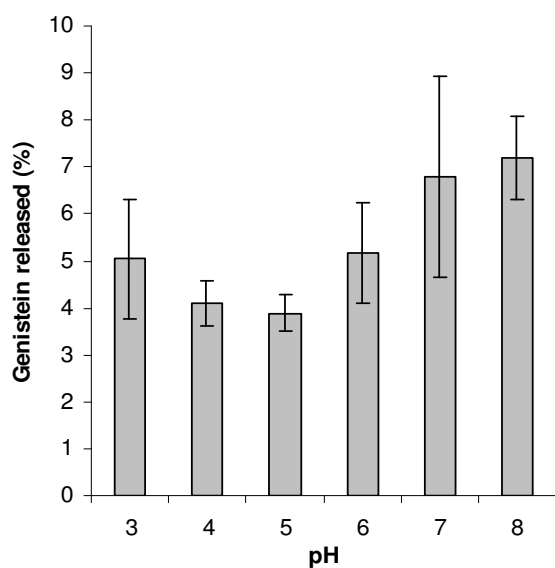

Figure 3: Release of genistein from amylose-genistein complexes under different pHs. Released genistein is expressed as percentage of total genistein in the complex. The release of genistein between pH = 4 and pH = 5 to pH = 8 is significant difference (P <0.05).
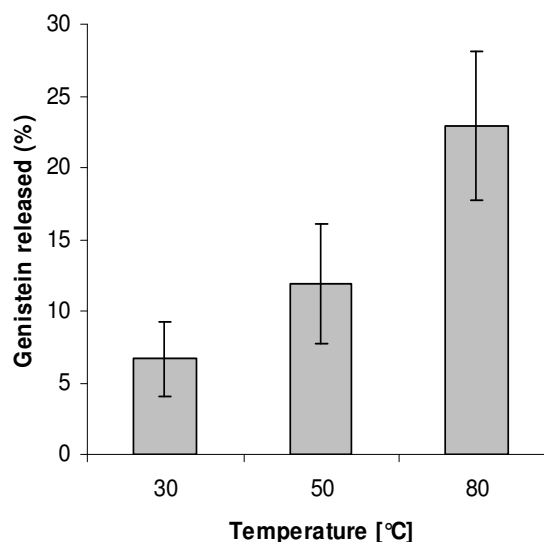
Figure 4: Release of genistein from amylose-genistein complexes at 30, 50 and 80 °C. Released genistein is expressed as percent out of total genistein in the complex. The release at the three temperatures is significantly different (P <0.05).

Because our efforts were focused on the possible use of these complexes as encapsulation matrix in food applications, we also tested the formation of these complexes by using high amylose corn starch (HACS). HACS-genistein complexes also exhibit a V6$_{III}$ structure under the XRD (Cohen et al., 2008). Genistein release from HACS complexes was tested by pancreatic digestion throughout a 24h period. From **Figure 6** it can be seen that genistein release in pancreatic solution is significantly higher than in PBS and that the maximum of genistein release is reached after 6h of incubation, which fits the normal transit time of food through the small intestine. Therefore, HACS-genistein particles produced by complexation can be proposed as a controlled and targeted release system, and the efficacy of this delivery system is to be tested *in-vivo* in the future.
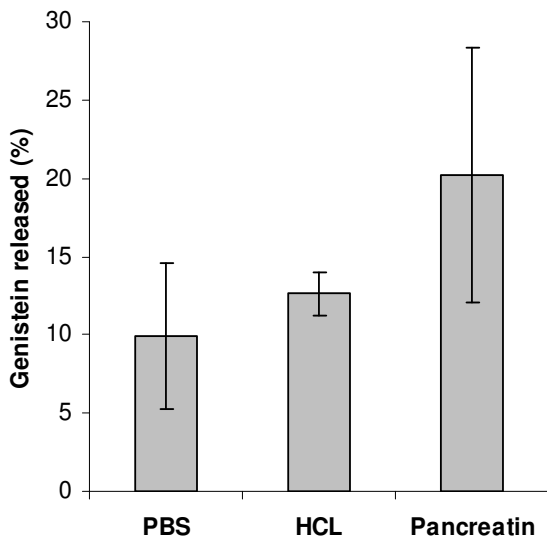
Figure 5: Release of genistein in simulated gastrointestinal conditions as compared to PBS. Genistein release is significantly higher by pancreatin (p<0.01).
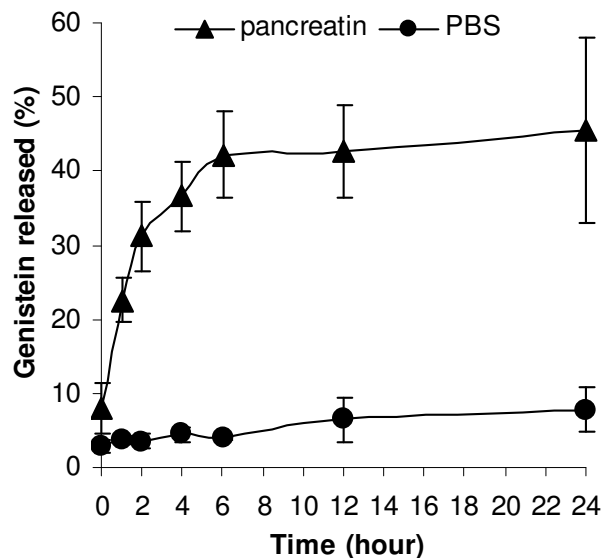

Figure 6: Release of genistein by pancreatic amylase over 24h from HACS complexes as compared to spontaneous release in PBS.

References

Biais, B., et al. (2006) *Structural and stoichiometric studies of complexes between aroma compounds and amylose.* Carbohydr. Polym., v.66, n.3, Nov 2, p.306-315.

Cohen, R., et al. (2008) *Structural and functional properties of amylose complexes with genistein.* J. Agric. Food Chem.

Conde-Petit, B., et al. (2006) *Structural features of starch-flavor complexation in food model systems.* Trends Food Sci. Tech., v.17, n.5, p.227-235.

Crupi, V., et al. (2007) *UV-vis and FTIR-ATR spectroscopic techniques to study the inclusion complexes of genistein with beta-cyclodextrins.* Journal of Pharmaceutical and Biomedical Analysis, v.44, n.1, May 9, p.110-117.

Griffith, A. P., et al. (2001) *Improved methods for the extraction and analysis of isoflavones from soy-containing foods and nutritional supplements by reversed-phase high-performance liquid chromatography and liquid chromatography-mass spectrometry.* J. Chromatogr. A, v.913, n.1-2, Apr 13, p.397-413.

Huang, A. S., et al. (1982) *Characterization of the non-volatile minor constituents responsible for the objectionable taste of defatted soybean flour.* J. Food Sci., v.47, n.1, p.19-23.

Kano, M., et al. (2006) *Bioavailability of isoflavones after ingestion of soy beverages in healthy adults.* J. Nutr., v.136, n.9, Sep, p.2291-2296.

Motlekar, N., et al. (2006) *Preparation and characterization of genistein containing poly(ethylene glycol) microparticles.* J. Appl. Polym. Sci. , v.101, n.3, Aug 5, p.2070-2078.