Food Research International 87 (2016) 42–49

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Modulating the gel properties of soy glycinin by crosslinking with tyrosinase

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ARTICLE INFO

Article history:
Received 3 April 2016
Received in revised form 14 June 2016
Accepted 18 June 2016
Available online 21 June 2016

Keywords:
Glycinin
Soy protein
Crosslinking
Tyrosinase
Gelation

ABSTRACT

The gelation progression and gel properties of enzymatically crosslinked soy glycinin were evaluated in comparison to non-crosslinked glycinin. Glycinin was initially crosslinked using tyrosinase from *Bacillus megaterium* (TyrBm) and was later used to form gel upon heating. Gelation was evaluated by small deformation rheological measurements and revealed a significant increase in storage modulus (G′) obtained in the crosslinked gel. This was confirmed by temperature sweep and frequency sweep measurements that supported the results and proved that the difference in modulus was not frequency dependent. Texture profile analysis showed an increase in hardness and decrease in elasticity of the crosslinked gels. Scanning electron microscopy (SEM) images displayed a more structural network with larger pore size in the crosslinked gel. The less dense structure of the crosslinked glycinin gel network led to a slight decrease in the water holding capacity. Finally, thermal analysis using differential scanning calorimetry (DSC) confirmed no change in the gelation point induced by denaturation, however thermal gravimetric analysis (TGA) did show a difference in the decomposition profile of the crosslinked protein compared with non-crosslinked glycinin. The results suggest that by applying TyrBm mediated crosslinking we may modulate the protein gel properties for tailoring the texture of food products.

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1. Introduction

With increasing awareness to the environment and the search for non-animal protein sources, soy proteins have become more popular and are being used in various food products as emulsifiers, gelation agents and protein additives (Friedman & Brandon, 2001; Hughes, Ryan, Mukherjea, & Schasteen, 2011; Liu, 1997; Mujoo, Trinh, & Ng, 2003; Renkema, Lakemond, de Jongh, Gruppen, & van Vliet, 2000; Thomson, Brinkworth, Noakes, & Buckley, 2016; Zhang, Wu, Lan, & Yang, 2013). They have a high nutritional value and are correlated with reduced LDL cholesterol uptake, prevention of diabetes and other health benefits (Friedman & Brandon, 2001; Zhang, Shu, Gao, et al., 2003). Glycinin is one of the storage soy proteins, which accounts for 30–35% of the total seed proteins. It is responsible for the gel properties of soy protein systems, and has been utilized and modified for various purposes such as Tofu production and milk substitutes (Liu, 1997; Mujoo et al., 2003; Utsumi, Matasumura, & Mori, 1997; Yasir, Sutton, Newberry, et al., 2007a, 2007b). Glycinin is a hexamer with a total molecular mass of ~350 kDa which comprises several sub-units. The glycinin monomer consists of an acidic polypeptide subunit (AS) with a size of ~38 kDa and a basic polypeptide subunit (BS) with a size of ~20 kDa, linked by a single disulfide bridge. At least six acidic polypeptides (A1a, A1b, A2–A4, and A5) and five basic polypeptides (B1a, B1b and B2–B4) have been isolated (Mujoo et al., 2003).

The properties and characteristics of glycinin gels obtained by heating have been widely investigated (Renkema, Knabben, & van Vliet, 2001; Renkema, Lakemond, de Jongh, et al., 2000). Other treatments have also been applied to induce gel formation of soy proteins, including acidification, conjugation with a carbohydrate and crosslinking by transglutaminase (Bhattacharya & Jena, 2007; Jian, Xiong, Guo, et al., 2014; Kang, Matsumura, Ikura, et al., 1994; Tang, Wu, Chen, & Yang, 2006; Zhang et al., 2013). Transglutaminase treatment on glycinin led to the formation of a strong, turbid gel at 40 °C. It was suggested however, that heat treatment might strengthen the gel due to formation of hydrophobic and hydrogen bonds as well as disulfide bonds that are involved in the gelation process of glycinin during heating (Tang et al., 2006). As the biological approach offers a higher specificity of the reaction preventing formation of side products, there is constant need for new and effective biological crosslinkers. This research offers a new enzymatic crosslinking mechanism by tyrosinase.

Bacterial tyrosinase from *Bacillus megaterium* (TyrBm) was previously characterized in our lab (Sendovski, Kanteev, Ben-Yosef, et al., 2011; Shuster & Fishman, 2009). TyrBm is a type-3-copper enzyme, which contains two copper ions in its active site that are necessary for its oxidation activity. It performs two successive reactions:

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http://dx.doi.org/10.1016/j.foodres.2016.06.018
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hydroxylation of monophenols and subsequent oxidation of the diphenols into quinones which polymerize spontaneously to form insoluble polymers such as melanin. Tyrosinases from bacterial or fungal origins have been investigated for their protein crosslinking ability on milk and wheat proteins (Heijnis, Wierenga, van Berkel, & Gruppen, 2010; Selinheimo, Autio, Kruus, & Buchert, 2007; Thalmann & Lötzbeyer, 2002). In previous work we have demonstrated the use of TyrBm crosslinking on glycinin to improve the properties of glycinin-stabilized oil-in-water emulsions (Isaschar-Ovdat, Rosenberg, Lesmes, & Fishman, 2015).

In the present study soy glycinin gels obtained after crosslinking with TyrBm were evaluated for their rheological behavior and texture properties. By introducing covalent bonds within the protein network we have modulated the gel properties and structure, allowing the potential use of this system in food applications.

2. Experimental

2.1. Materials

Soy glycinin standard (98% purity), sodium bisulfite and all other chemicals were obtained from Sigma Chemical Co. (Rehovot, Israel). Tissue freezing medium for the SEM samples was purchased from TED PELLA Inc. (Redding, CA, USA). Defatted soybean flakes were kindly provided by Shemen Industries Ltd. (Haifa, Israel). Protein molecular weight marker was purchased from m.biotech (Hanam, Korea).

2.2. Methods

2.2.1. Purification of TyrBm and activity determination

TyrBm was isolated as previously described (Isaschar-Ovdat et al., 2015).

2.2.2. Isolation of soy protein fractions

Glycinin-rich fraction was isolated from defatted soybean flakes as previously described (Isaschar-Ovdat et al., 2015).

2.2.3. Enzymatic crosslinking of soy glycinin

Glycinin at 2% (w/v) was suspended in 50 mM Tris-HCl buffer pH 7.5 (reaction buffer) for 30 min at ambient temperature and TyrBm was added at 1:25 ratio (0.08% w/v) (Isaschar-Ovdat et al., 2015). The non-crosslinked glycinin was treated similarly but without the enzyme. The reaction mixtures were incubated at 37 °C with shaking at 250 rpm in an incubator shaker (TU-400 Orbital Shaker Incubator, MRC, Holon) for 4 h, then stopped by adding ethylenediaminetetraacetic acid (EDTA) to a final concentration of 1.5 mM, which was previously shown to inhibit TyrBm by chelation of copper (unpublished results). The reaction mixtures were lyophilized and kept at −20 °C until further use.

2.2.4. SDS-PAGE analysis

SDS-PAGE was performed on a discontinuous buffered system (Laemmli, 1970) using 12% separating gel and 4% stacking gel. The samples were heated for 5 min at 95 °C, after adding reducing sample buffer (4×), 1:1 (v/v). The gel was stained with 0.25% Coomassie brilliant blue (R-250) in 50% ethanol and 10% acetic acid, and destained in 10% acetic acid [methanol:acetic acid:water, 20:10:70 (v/v/v)].

2.2.5. Preparation of heat-induced gels

Gel samples (5 mL) were prepared by mixing 10% (w/v) non-crosslinked or crosslinked glycinin dispersion with distilled H_2O in sealed glass cylinders (15 mm diameter, 50 mm height). The mixture was incubated for 30 min at 95 °C followed by cooling under running tap water and overnight at 4 °C. Before measurements the gels were equilibrated to room temperature for 30 min.

2.2.6. Thermal gravimetric analysis of dried non-crosslinked and crosslinked glycinin

Thermal gravimetric analysis (TGA) was performed using TGA-Q5000 system (TA Instruments, USA). Analysis was done on the lyophilized non-crosslinked or crosslinked protein powder. Samples were heated under nitrogen atmosphere from room temperature to 600 °C at a heating rate of 10 °C·min^{-1}. The results were analyzed using Universal Analysis 200 version 4.5A build 4.5.0.5 software.

2.2.7. Differential scanning calorimetry

The protein denaturation was analyzed by differential scanning calorimetry (DSC) equipped with a HSS7 high-sensitivity sensor (DSC1 system, Mettler-Toledo, USA). Measurements were carried out under a nitrogen atmosphere. 45 μL of 10% (w/v) non-crosslinked or crosslinked glycinin dispersion in medium buffer were loaded into a sealed stainless steel pan to prevent mass loss during the experiment. An empty, hermetically sealed stainless steel pan was used as the reference. The samples were scanned from 25 to 150 °C at 5 °C·min^{-1}. The temperature at which denaturation starts, the onset temperature (T_o), was calculated by taking the intercept of the baseline and the extrapolated slope of the peak. The peak denaturation temperature (T_p) was taken as the temperature of maximum heat flow. The enthalpy of denaturation (ΔH) was integrated by the peak area between the starting point of transition to the end point of transition. Values presented are the mean ± SD (standard deviation) of 7 repetitions.

2.2.8. Rheological measurements at small deformation

Rheological measurements using parallel plates ($d = 40$ mm) were carried out in a Discovery Hybrid Rheometer (DHR-2, TA Instruments, DE, USA). 10% (w/v) non-crosslinked or crosslinked glycinin were dispersed in reaction buffer and stirred at 4 °C overnight. The mixture was equilibrated to room temperature for 30 min before analysis. The sample was placed between parallel plates at 25 °C and the gap between the two plates was set to 1.0 mm. The temperature was monitored through the lower plate. Excess sample was trimmed and exposed edges were covered with a thin layer of silicone oil to prevent solvent evaporation. The equipment was controlled using the Trios program (TA Instruments, DE, USA).

2.2.8.1. Temperature sweep measurements of non-crosslinked and crosslinked glycinin gels. Temperature sweep curves were recorded, after reaching equilibrium, by heating from 25 °C to 95 °C at 5 °C·min^{-1}, holding for 30 min at 95 °C and then cooling back to 25 °C at the same rate and holding for 15 min at 25 °C. The storage modulus (G′) and loss modulus (G″) were recorded as a function of time. Experiments were conducted using a fixed frequency of 0.1 Hz and 4 Pa stress within the linear viscoelastic region (LVR) (determined prior at the relevant temperature range).

2.2.8.2. Frequency sweep measurements of non-crosslinked and crosslinked glycinin. The storage modulus (G′) and loss modulus (G″) were recorded as a function of frequency. Frequency sweep measurements were performed using a frequency range of 0.01–10.0 Hz with a constant strain of 2% within the LVR region (determined prior at the relevant temperature range). Frequency sweep experiments were conducted at different temperatures using 5 °C intervals during heating and cooling between 25 and 95 °C. The samples were manually heated or cooled to the desired temperature and a frequency sweep experiment was conducted.

2.2.9. Textural profile analysis (TPA) of non-crosslinked and crosslinked glycinin gels

The textural characteristic of the prepared gels was analyzed according to a texture profile analysis (TPA) using a Texture Analyzer (Model LRX-5 K, Lloyd Instruments, UK). The gel samples were prepared in a glass cylinder with dimensions of 15 mm diameter × 50 mm height and remained in the glass cylinder during the measurement. The

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