



Interactions between tea polyphenol and two kinds of typical egg white proteins—ovalbumin and lysozyme: Effect on the gastrointestinal digestion of both proteins in vitro

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ABSTRACT

The promotion or inhibition of gastrointestinal digestion of tea polyphenol (TP) towards the two typical proteins from egg white (ovalbumin (OVA) and lysozyme (LYZ)) was examined. The results showed that TP made OVA/LYZ easier for digestion in the pepsin solution at pH 1.2 and inhibited OVA/LYZ digestion in pancreatin solution at pH 7.5. Non-covalent interactions between OVA/LYZ and TP and the secondary structure of OVA/LYZ were studied by using Fluorescence spectroscopy and Fourier transform infrared spectroscopy (FTIR), respectively. Results suggested that stronger conformational change occurred at pH 1.2 compared with that of pH 7.5 affected by TP in both proteins. Non-covalent interactions between OVA/LYZ and TP at pH 1.2 increased random and β -sheet structures in both proteins at the expense of α -helix, which resulted in the proteins with looser structures. At pH 7.5, an opposite second structural change of both proteins caused by the non-covalent interactions between OVA/LYZ and TP. The conformational and second structural change of proteins (substrate) might be a reason for promoting and inhibiting digestion of OVA/LYZ affected by TP.

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1. Introduction

Egg and tea-leaf often combined to make many foods such as delicious green tea cakes and traditional Chinese tea-leaf eggs. Tea-leaf can endow eggs or egg products with antibacterial property, antioxidant property, color and flavors (Chen, Lu, Chien, & Chen, 2010; Lu, Lee, Mau, & Lin, 2010), yet it had received considerable attention in recent years for the possibility of affecting the digestion of proteins due to an abundant amount of tea polyphenol (TP) in tea-leaf (He, Lv, & Yao, 2007; Stojadinovic, Radosavljevic, Ognjenovic, Vesic, Prodic, Stanic-Vucinic, et al., 2013; Tagliazucchi, Verzelloni, & Conte, 2005; Tantoush, Apostolovic, Kravic, Prodic, Mihajlovic, Stanic-Vucinic, et al., 2012).

There was a dispute about the effects of polyphenols on protein digestion. One view was that polyphenols would increase the velocity of the enzymatic reaction and the activating effect was concentration dependent (Tagliazucchi et al., 2005; Tantoush et al., 2012). The other researchers demonstrated that polyphenols had inhibitory effects on α -amylase, pepsin, trypsin and lipase and slowed down gastrointestinal digestion of proteins such as β -lactoglobulin (He et al., 2007; Stojadinovic et al., 2013). Though efforts have been made to explore

the effects of polyphenols on protein digestion, the effects of TP on proteins (especially OVA and LYZ) digestion were still unknown.

TP, the major ingredient of tea, accounts for up to 30% of the tea dry weight (Lin, Juan, Chen, Liang, & Lin, 1996). It has been reported to affect the gastrointestinal digestion of proteins (McDougall & Stewart, 2005; Record & Lane, 2001; Tantoush et al., 2012). The major protein in chicken egg white is ovalbumin (OVA), comprising 60–65% of total egg white proteins (Huntington & Stein, 2001). While lysozyme (LYZ), of which the content is not very high, is a specific basic protein in egg white (Blake, Koenig, Mair, North, Phillips and Sarma, 1965). For this reason, OVA and LYZ are chosen as representative proteins of egg white to explore promoting or inhibiting effect of TP on proteins in the gastrointestinal digestion which is based on in vitro experiments.

Previously, many researchers have tried to find the impact mechanism of TP on protein digestion. Most of them concentrated on the TP-enzyme and few considered the effect of TP-substrate while it is a common sense in the field of Biochemistry that the ratio of K_{cat} (turnover number)/ K_m (K_m is the substrate concentration that lets the reaction occur at $1/2 V_{max}$. V_{max} is the maximum rate of the reaction when the substrate concentration is very high) is defined as a measure of the catalytic efficiency of an enzyme–substrate pair (Berg, Tymoczko, & Stryer, 2002). Changing the substrate structure could impact the enzyme target site, and then affect the efficiency of enzyme-catalyzed reaction (Berg et al., 2002). Non-covalent interactions between polyphenols and proteins may affect the stabilization of protein

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secondary structure (Kanakis, Hasni, Bourassa, Tarantilis, Polissiou and Tajmir-Riahi, 2011), protein unfolding, precipitation and conformation (Papadopoulou & Frazier, 2004; Rothwarf & Scheraga, 1996; Siebert, Troukhanova, & Lynn, 1996). Therefore, addressing the interactions of proteins and polyphenols to attempt the elucidation of structure–activity relationships was a major step to elucidate the TP-induced mechanism of gastrointestinal digestion of OVA/LYZ.

It is a well-recognized fact that the intrinsic fluorescence is widely applied to detect the changes in microenvironment of the Trp residues in the proteins (Dufour & Dangles, 2005; Ghosh, Sahoo, & Dasgupta, 2008; Hui, Quan, Jian, Jianbo, & Ming, 2008; Wang, Yin, Li, Wang, Pu, Wang, et al., 2013; Xiao, Cao, Wang, Yamamoto, & Wei, 2010), and fluorescence spectroscopy is an appropriate method to determine the binding parameters between TP and proteins (H. M. Rawel & Frey, 2006). FTIR technique for the structural characterization is also well addressed in the literature during the past several decades (Byler & Susi, 1986; Prestrelski, Fox, & Arakawa, 1992; Surewicz, Mantsch, & Chapman, 1993).

In this study, gastrointestinal digestion of OVA/LYZ in the presence of TP was simulated. Binding affinity between TP and OVA/LYZ and TP-induced protein structural change were also studied at different pH values (about 1.2 in the stomach and 7.5 in the intestine and saliva; Pharmacopeia, 1985). Fluorescence quenching spectroscopy was used to elucidate the binding affinity between TP and proteins, and the secondary structure of proteins was determined by FTIR. The objectives of this study were to evaluate the effects of TP on protein digestion and to clarify their impact mechanism.

2. Materials and methods

2.1. Materials

OVA (from egg white), LYZ (from egg white, twice crystalline), and all other chemicals (reagent grade), were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, P.R. China), unless otherwise noted. Tea polyphenol (>98%) was obtained from 3 W Botanical Extract Inc. (Hunan, China). Pepsin (from porcine stomach mucosa, 600 U/mg) and pancreatin (from bovine pancreas, 2500 U/mg) were both purchased from Sigma-Aldrich (Taufkirchen, Germany).

2.2. *In vitro* pepsin digestibility assay

In vitro simulated gastrointestinal digestion was performed following the method described by Stojadinovic et al. (2013) with some modifications (Stojadinovic et al., 2013). Briefly, 180 μ L of OVA (5 mg/mL), added with or without 150 μ L of TP solutions (6 mg/mL) at a ratio of OVA/TP (1:1 w/w), was diluted with 330 μ L of deionized water and 240 μ L of 4 $\times$ simulated gastric fluid (SGF) (0.2 M HCl with 8 g/L NaCl, pH 1.2), then the solutions were warmed at 37 °C and the pH was adjusted to 1.2 with 0.5 M HCl if necessary. Prewarmed 300 μ L of 0.05 mg/mL pepsin in 0.02 M HCl, 0.4 g/L NaCl pH 1.2 buffer was added to each reaction mixture. Final ratio of pepsin units to mg of protein was 10. The digestion was performed at 37 °C in incubator with moderate agitation, and aliquots (80 μ L) were taken at 0.25, 0.5, 1, 2, 3 and 6 h for further analysis. The digestion was stopped by 11 μ L of 2 M Na₂CO₃ and 22 μ L of five times concentrated electrophoresis sample buffer containing reducing agent 2-mercaptoethanol for 15% SDS-PAGE analysis and the bands obtained were analyzed by densitometry. OVA control was prepared with the addition of deionized water instead of the TP and pepsin solution. Pepsin digestibility test of LYZ was done in the same manner as OVA, but the concentration was twice the amount of it to facilitate analysis. Dose–response experiments of digestion (the ratio of protein and TP used in the assay was 1:0, 1:0.25, 1:0.5, 1:1, 1:2, 1:4, 1:8, w/w) were done as previously written, but the digestion was stopped in 3 h. All digestions were done in duplicates.

2.3. *In vitro* pancreatin digestibility assay

Mixture containing 180 μ L of OVA (0.5 mg/mL) and 150 μ L TP solutions (6 mg/mL), the same as described in the pepsin digestion, in 20 mM sodium phosphate buffer pH 7.5 was prewarmed at 37 °C, then 300 μ L of prewarmed pancreatin solution (0.12 mg/mL) and 100 U pancreatin U/mg^{−1} of proteins, in 80 mM sodium phosphate buffer, pH 7.5 were added. Digestion proceeded at 37 °C in closed tubes with vigorous shaking. Aliquots (80 μ L) were taken at 0.25, 0.5, 1, 2, 3 and 6 h and stopped by adding 22 μ L of 5 times reducing sample buffer and boiling for 5 min at 95 °C. 10 μ L of each sample was analyzed by SDS-PAGE and densitometry. OVA control was prepared by adding a mixture of OVA and pancreatin to prewarmed 80 mM sodium phosphate buffer, pH 7.5. Control digestion was performed in the same manner but without TP. Pancreatin digestibility test of LYZ was done in the same manner as OVA, but the concentration was twice the amount of it to facilitate analysis. Dose–response experiments were done as pepsin digestibility assay. All digestions were done in duplicates.

2.4. SDS-PAGE

10 μ L of each sample taken and treated in different stages of the digestion was analyzed by SDS-PAGE (Laemmli, 1970). Specific steps are as follows: the samples were mixed with the sample buffer (0.5 mol L^{−1} Tris–HCl, pH 6.8, containing 40 g L^{−1} SDS, 200 mL L^{−1} glycerol and 100 mL L^{−1} β -ME) at a ratio of 1:1 (v/v) and boiled for 3 min. The samples (10 μ L protein) were loaded onto the polyacrylamide gel made of 15% running gel and 4% stacking gel and subjected to electrophoresis at 80 V (until the bromophenol blue band pass the stacking gel) and 120 V (until the bromophenol blue band pass the separating gel) per gel. After electrophoresis, the gel was stained with 0.2 g L^{−1} (w/v) Coomassie Brilliant Blue R-250 in 500 mL L^{−1} (v/v) methanol and 75 mL L^{−1} (v/v) acetic acid and destained with 500 mL L^{−1} methanol (v/v) and 75 mL L^{−1} (v/v) acetic acid, followed by 50 mL L^{−1} methanol (v/v) and 75 mL L^{−1} (v/v) acetic acid. Molecular weight markers (Sigma Chemical Co.) were used to estimate the molecular weight of proteins.

2.5. Densitometry analysis

All gels were analyzed in Image Lab 3.0 program (Bio-Rad, California, USA), using lane/band and quantitative tool option. The bands of interest were outlined with the lane/band tool and the option lanes/bands were chosen. A monochrome image was created for the purpose of the analysis. The lanes and density centers of the bands were found automatically or manually. The intensity of each band was presented relative to the band in the control lane designated as 100%. The relative amount of sample at different stages helped to know the kinetic model of digestion calculated by Origin 8.0 program (Northampton, MA, USA). The T₅₀ was the time that half of the protein was digested, and T₉₀ was the time that 90% of the protein was digested.

2.6. Fluorescence spectroscopy

Fluorescent spectra were recorded on an F-7000 fluorescence spectrophotometer (Hitachi, Japan) in a 1 cm path length quartz cell. Two buffers were used: 0.1 M HCl with 2 g/L NaCl pH 1.2 and phosphate buffered saline (PBS) pH 7.5. Three milliliter proteins (0.05 mg/mL) were titrated by successive additions of a 50 μ L stock solution of TP (from 0.3 mg/mL to 3 mg/mL) with sufficient mixing. The fluorescence spectra were immediately obtained (excitation at 280 nm and emission wavelengths of 290–410 nm) at room temperatures. The blank spectrum was automatically subtracted from the emission spectrum of the corresponding solution. Spectra were further analyzed using an Origin 8.0 program.

2.7. Fluorescence quenching analysis

Fluorescent quenching was described by Stern–Volmer equation:

$$F_0/F = 1 + K_q \tau_0 [Q] = 1 + K_{SV} \times [Q] \quad (1)$$

(H. A. Rawel, Meidtnr, & Kroll, 2005)

where K_q , τ_0 , K_{SV} , and $[Q]$ are the quenching rate constant of the bimolecular, the average lifetime of the protein without quencher, the Stern–Volmer dynamic quenching constant, and the concentration of the quencher, respectively. F_0 and F are the fluorescence intensities before and after the addition of the quencher, respectively. A linear Stern–Volmer plot indicated that one mechanism of quenching occurred static (complex formation) or dynamic. The maximum value possible of K_q for diffusion-limited quenching in water was $2.0 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ (Ware, 1962; Zhang, Zhou, Liu, Zhou, Ding & Liu, 2008). When the rate constants of protein quenching procedure initiated by the quencher (TP) were greater than that of diffusion-limited quenching, it could mean that there was a complex formation between a protein and a quencher, corresponding to a static mechanism of the fluorophore quenching. In the static quenching process, the number of binding places (n) and binding constant K_a could be calculated according to the following equations, respectively:

$$\log[(F_0 - F)/F] = \log K_a + n \log [Q] \quad (2)$$

(Lakowicz, 2009)

$$(F_0 - F)^{-1} = F_0 + K_a^{-1} F_0^{-1} [Q]^{-1} \quad (3)$$

(Lakowicz, 2009).

The slope of the double logarithmic Stern–Volmer (Eq. (2)) plot yielded the number of binding sites and the slope of Eq. (3) provided the binding constant (K_a). All plots were created and analyzed in OriginPro 8. Every experiment was repeated three times and statistical analysis was performed using one way ANOVA followed by Bonferroni test (compared selected pairs of samples).

Table 1

Estimated pepsin and pancreatin digestion $T_{50\%}$ and $T_{90\%}$ of OVA and LYZ in the absence and presence of TP.

Protein/treatment	Pepsin		Pancreatin	
	$T_{50\%}$ (min)	$T_{90\%}$ (min)	$T_{50\%}$ (min)	$T_{90\%}$ (min)
OVA	29 ± 2b	315 ± 10c	10 ± 1a	26 ± 2a
OVA + TP (1:1)	14 ± 0a	169 ± 10b	11 ± 2a	48 ± 2a
LYZ	49 ± 4c	165 ± 3b	36 ± 5b	94 ± 1b
LYZ + TP (1:1)	12 ± 1a	45 ± 1c	153 ± 6c	>360c

Values are expressed as mean ± standard error. All values within a column with different letters are significantly different, $p < 0.05$

2.8. FTIR spectroscopic measurements

Infrared spectra of OVA-TP and LYZ-TP were recorded on an FTIR spectrometer (Nicolet iS10), equipped with deuterated triglycine sulfate (DTGS) detector and KBr beam splitter, using ZnSe windows. Protein concentration was 20 mg/mL while TP concentration was 4 and 20 mg/mL. Samples were prepared in three different buffers of pH 1.2 (0.1 M HCl, 2 g/L NaCl) and pH 7.5 (20 mM sodium–phosphate), and followed by vacuum freeze drying to dry powder. Spectra were recorded in a range of 4000–600 cm^{-1} to investigate the structural conformation of proteins by adding TP. Blank KBr beam splitter was used to adjust to the baseline level each time before measurement, in order to normalize difference spectra. All tests were done in duplicates.

2.9. Analysis of protein conformation

Analysis of the secondary structure of OVA/LYZ and its polyphenol complexes was carried out on the basis of the procedure already reported (Byler & Susi, 1986; Choi & Ma, 2005; Sun, Zhou, Zhao, Yang, & Cui, 2011). Deconvolution spectra were obtained by Omnic 8.0 software (Thermo Fisher Scientific Inc. Waltham, MA). The half-bandwidth used for deconvolution was 13 cm^{-1} , and the enhancement factor was 2.4. To ensure that the spectra were not over-deconvoluted, the acquired spectra were judged by evaluating the second derivative spectra, comparing the number and positions of the bands with those of the deconvoluted spectra. Quantitative estimation of secondary structure components was performed using Gaussian peaks and curve fitting models according to Byler and Susi (Byler & Susi, 1986).

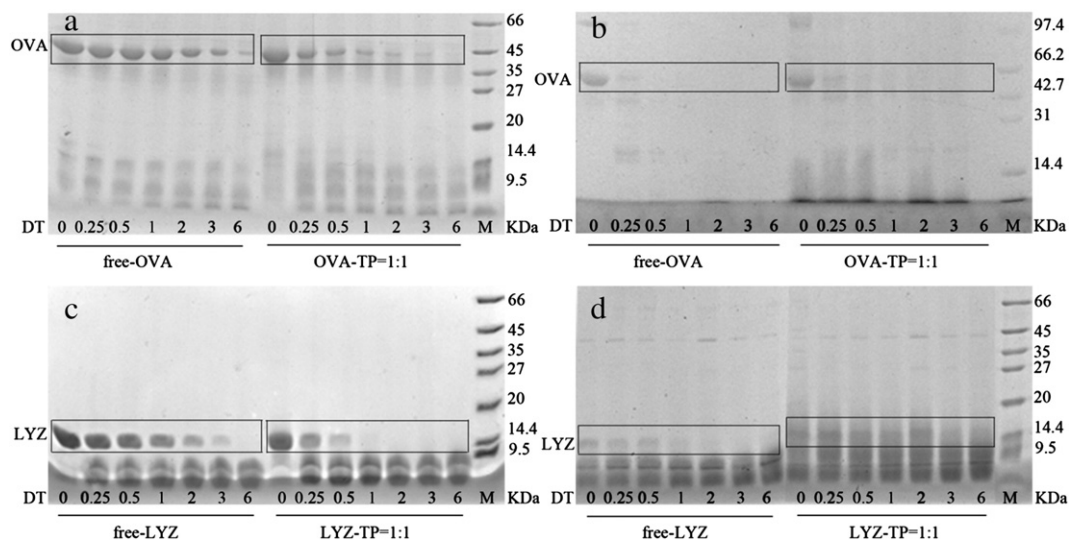


Fig. 1. SDS-PAGE analysis of the digestion of OVA/LYZ with and without TP in simulated gastric and intestinal digestions (a) free-OVA and TP-OVA pepsin digestion, (b) free-OVA and TP-OVA pancreatin digestion, (c) free-LYZ and TP-LYZ pepsin digestion, and (d) free-LYZ and TP-LYZ pancreatin digestion; DT — digestion time; 0, 0.25, 0.5, 1, 2, 3, and 6: digestion time of 0 h, 0.25 h, 0.5 h, 1 h, 2 h, 3 h, and 6 h; free-OVA/LYZ: without TP; TP-OVA/LYZ (1:1): the ratio of OVA/LYZ and TP used in the assay is 1:1, w/w. M — molecular weight marker proteins.

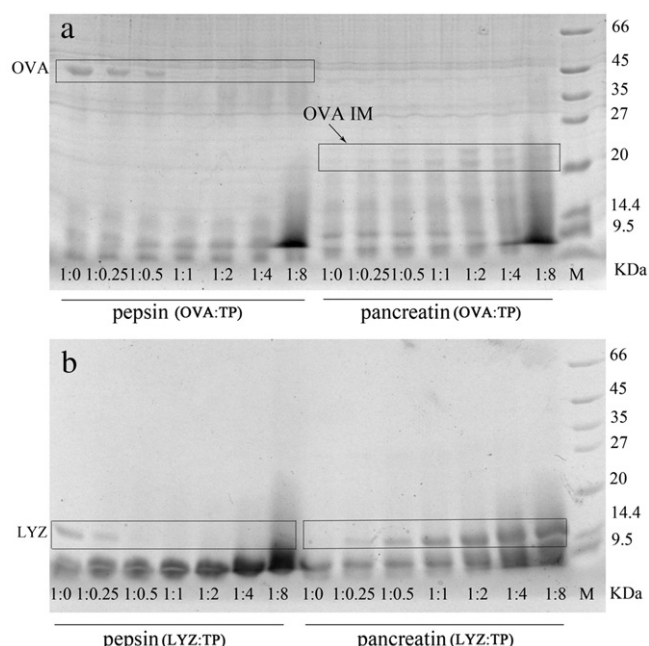


Fig. 2. TP facilitated pepsin digestion and hampered pancreatin digestion of OVA/LYZ. The effect of increased concentrations (the ratio of protein and TP used in the assay is from 1:0 to 1:8, w/w) of TP on OVA/LYZ gastrointestinal digestion is analyzed by SDS-PAGE. The effect of TP on (a) OVA and (b) LYZ gastrointestinal digestion. M – molecular weight marker proteins. OVA IM – OVA intermediate in the enzymatic hydrolysis.

3. Results

3.1. Effects of TP on OVA and LYZ digestibility

The digestion of OVA and LYZ in the absence and presence of TP was monitored by SDS-PAGE. Promoting effect of pepsin digestion and inhibiting effect of pancreatin digestion on both proteins influenced by TP could be visually shown in Fig. 1. Proteins decay following the

first-order kinetics (Ziyad Tantoush et al., 2012), allowing estimation of $T_{50\%}$ and $T_{90\%}$ under all tested conditions. The results obtained were shown in Table 1. In simulated gastric fluid (SGF) conditions, TP made OVA/LYZ easier for digestion in the pepsin solution at pH 1.2, while an opposite result was found in simulated intestinal fluid (SIF) conditions.

In the control reaction group, under the SGF conditions, the T_{90} of OVA and LYZ is 315 min and 165 min, respectively (Table 1). In the presence of TP (protein:TP ratio 1:1, w/w), the T_{90} of OVA is shorter (169 min), so does the LYZ (45 min), suggesting that both proteins were easier to be digested by pepsin in the presence of TP. The same conclusion could be drawn from Fig. 2a and b, which showed that with the increasing of TP addition, the electrophoresis stripes of OVA and LYZ are lighter.

While the in vitro intestinal digestion of proteins (OVA, LYZ) presented opposite results: with the presence of TP (protein:TP ratio 1:1, w/w), the T_{90} of OVA (26 min) and LYZ (94 min) is increased to 48 min and 360 min, respectively (Table 1). Fig. 2a showed that OVA had all been digested after 3 h, but electrophoresis stripes of the intermediate in the end of the enzymatic hydrolysis became wider and darker with increasing TP concentrations which agreed with the result in Table 1. Fig. 2b obviously showed that the electrophoresis stripes of the remaining LYZ became wider and darker with the increasing of TP concentrations, which also consist with the result in Table 1. These results suggested that OVA and LYZ became more difficult to be digested by pancreatin with the addition of TP.

3.2. Effect of TP on protein fluorescence spectra

The fluorescence spectra of OVA/LYZ added with TP were shown in Fig. 3. Addition of TP decreased fluorescence intensities of proteins. The blue shifts of the maximum λ_{em} fluorescence of OVA and LYZ were observed at both pH 1.2 and pH 7.5. The maximum λ_{em} of OVA at pH 1.2 shifts from 333 cm^{-1} to 327 cm^{-1} and the blue shift is about 9 cm^{-1} , while the blue shift at pH 7.5 is about 5 cm^{-1} from 335 cm^{-1} to 330 cm^{-1} . The blue shift of LYZ at pH 1.2 and pH 7.5 is about 11 cm^{-1} (341 cm^{-1} to 330 cm^{-1}) and 3 cm^{-1} (340 cm^{-1} to 337 cm^{-1}), respectively (Fig. 3). Blue-shifts indicated the

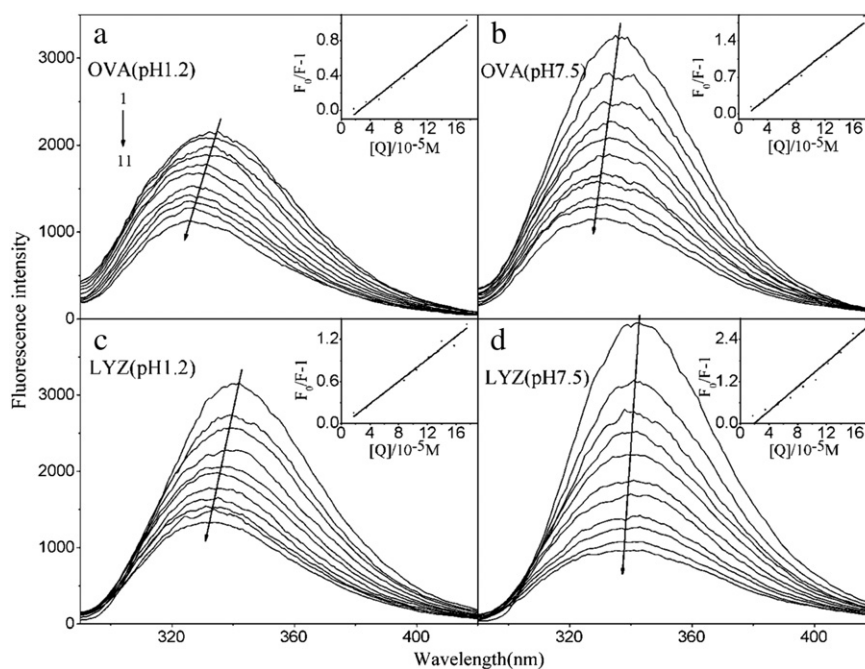


Fig. 3. The quenching effects of TP on OVA/LYZ intrinsic fluorescence at pH 1.2 and 7.5. Fluorescence spectra of OVA/LYZ without (1) and with the addition of TP (2–11) at pH 1.2 and 7.5. 1: 0 $\mu\text{g/mL}$ TP; 2: 5 $\mu\text{g/mL}$ TP; 3: 10 $\mu\text{g/mL}$ TP; 4: 15 $\mu\text{g/mL}$ TP; 5: 20 $\mu\text{g/mL}$ TP; 6: 25 $\mu\text{g/mL}$ TP; 7: 30 $\mu\text{g/mL}$ TP; 8: 35 $\mu\text{g/mL}$ TP; 9: 40 $\mu\text{g/mL}$ TP; 10: 45 $\mu\text{g/mL}$ TP; 11: 50 $\mu\text{g/mL}$ TP; at pH 1.2 and 7.5. Insets: the Stern–Volmer plots of $F_0/(F_0-F)$ at 340 nm and room temperature as function of concentration of quencher as per Eq. (1).

Table 2

Calculated quenching rate constant (K_q), binding constants (K_a) for the OVA/LYZ-TP complexes and the number of TP bound per a molecule of protein (n).

	K_q	R_1	K_a	R_2	n	R_3
OVA (pH = 1.2)	8.72×10^{12}	0.987	817.39	0.932	1.767	0.984
OVA (pH = 7.5)	3.39×10^{12}	0.986	7898.89	0.999	1.134	0.996
LYZ (pH = 1.2)	1.06×10^{13}	0.981	7939.03	0.964	1.001	0.983
LYZ (pH = 7.5)	5.54×10^{12}	0.965	12846.80	0.981	1.142	0.972

R_1 , R_2 , and R_3 are the correlation coefficients for K_q , K_a , and n , respectively.

movement of tyrosine and tryptophan residues of proteins to hydrophobic region during the interactions between OVA/LYZ and TP (Zakin & Herschbach, 1986), thus causing conformational change of both proteins (Papadopolou, Green, & Frazier, 2005; Wang et al., 2013). The degree of blue shifts at pH 1.2 is much greater than that of pH 7.5 with the addition of TP, which might imply a stronger conformational change that occurred at pH 1.2 compared with that of pH 7.5 on both proteins.

Previous researches indicated that a linear Stern–Volmer plot represents a single quenching mechanism, either static or dynamic (Shoemaker, Garland, Nibler, & Feigerle, 1967). Thereinto, in a static quenching process, generally, a linear Stern–Volmer plot indicates either only one drug binding site in the proximity of fluorophore exists, or more than one binding site all equally accessible to quenchers (Yang, Hu, Fan, & Shen, 2008). As shown in the inset of Fig. 3, the plot of $F_0/F-1$ for proteins versus $[Q]$ at low concentrations of TP exhibits linearity, and every K_q (based on Eq. (1)) at different pHs is in the range of 10^{12} – 10^{13} . Obviously, the value for K_q is 2 to 3 orders of magnitude greater than the maximum diffusion collision quenching rate constant ($2.0 \times 10^{10} \text{ Lmol}^{-1} \text{ s}^{-1}$) for a variety of quenchers (Yang et al., 2008). Therefore, it indicated that the fluorescence quenching process of both

proteins might be mainly governed by a static quenching mechanism rather than a dynamic quenching mechanism. This result agreed with the conclusion of Rui-qiang Wang et al. (2013).

In TP-OVA/LYZ system, the values of K (binding constant) and n (number of binding places) at different pHs could be derived from the slopes of plots (Fig. 3) (based on Eqs. (2) and (3)), and both values were presented in Table 2. Table 2 showed that TP exhibited a stronger affinity with OVA/LYZ at pH 7.5 than at pH 1.2. Binding constant K between TP and OVA at pH 7.5 (7899) is as about ten times as at pH 1.2 (817). LYZ reveals the similar performance of binding constant K , 7939 (pH 1.2) and 12,847 (pH 7.5). Our results are similar with the results of Papadopolou et al., which showed that flavonoids exhibited a stronger affinity to proteins at pH 7.4 than that of lower pH (Papadopolou et al., 2005). The reason for the strength of affinity might be the electrostatic interaction between TP and OVA/LYZ. Since OVA/LYZ (isoelectric point (pI) for OVA is 4.6 and pI for LYZ is 11.0 (Hagerman & Butler, 1978)) and TP (pKa = 9.0–10.0 (Hider, Liu, & Khodr, 2001)) carry a similar net positive charge at pH 1.2, the repulsive force between TP and proteins is strong, and the strength of affinity is low. However, at pH 7.5, OVA and TP carry net negative charges and positive charges, respectively so that electrostatic attraction occurred. While LYZ carried less positive charges (pH 7.5) than that of pH 1.2, the electrostatic exclusive is weakened, and the strength of affinity is high.

3.3. FTIR spectra of TP-OVA/LYZ complexes

The TP-OVA/LYZ complexation was characterized by infrared spectroscopy and its derivative methods. The original infrared spectrums of free-protein and TP-protein were shown in Fig. 4. From Fig. 4 we could find that there is more or less spectral shifting for the protein

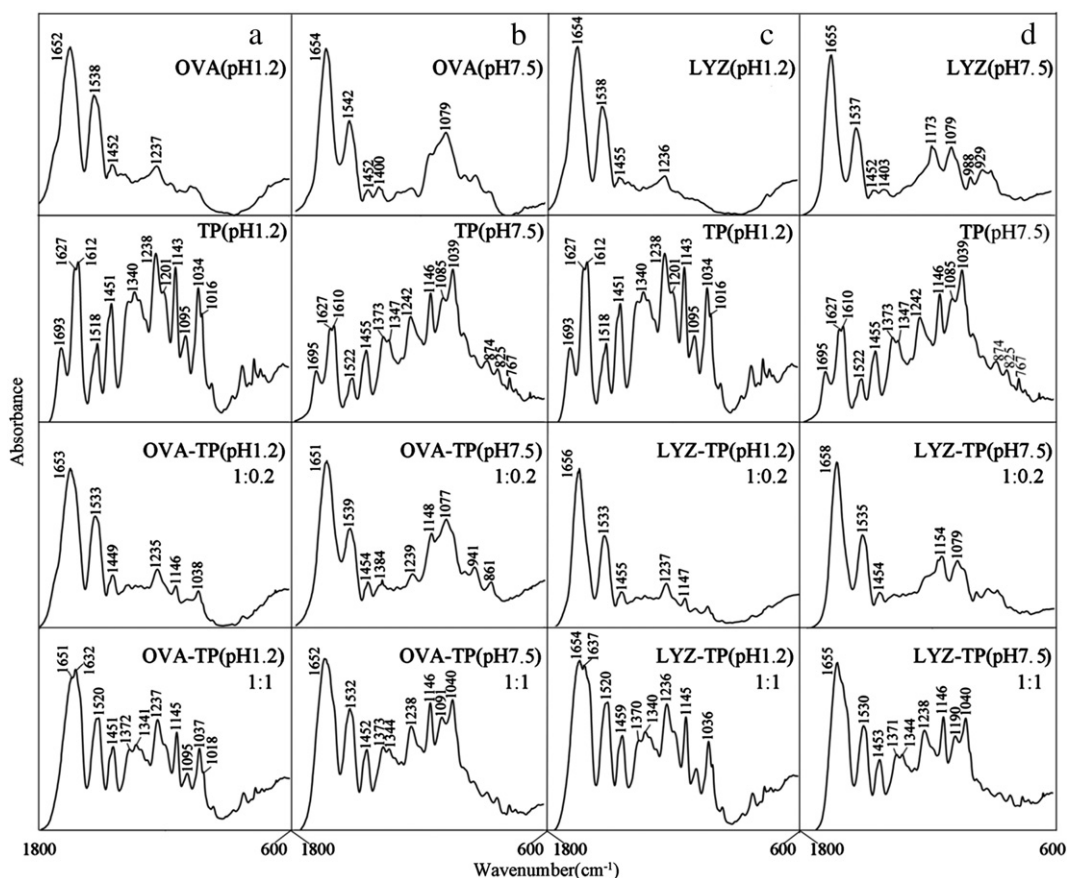


Fig. 4. FTIR spectra in the region of $1800\text{--}600 \text{ cm}^{-1}$ of KBr beam splitters for free OVA (pH 1.2) (a), free OVA (pH 7.5) (b), free LYZ (pH 1.2) (c), free LYZ (pH 7.5) (d), and free TP (pH 1.2 and pH 7.5) with difference spectra of OVA/LYZ-TP complexes (bottom eight curves) obtained at different TP concentrations (the ratio of protein and TP used in the assay are 1:0.2 and 1:1).

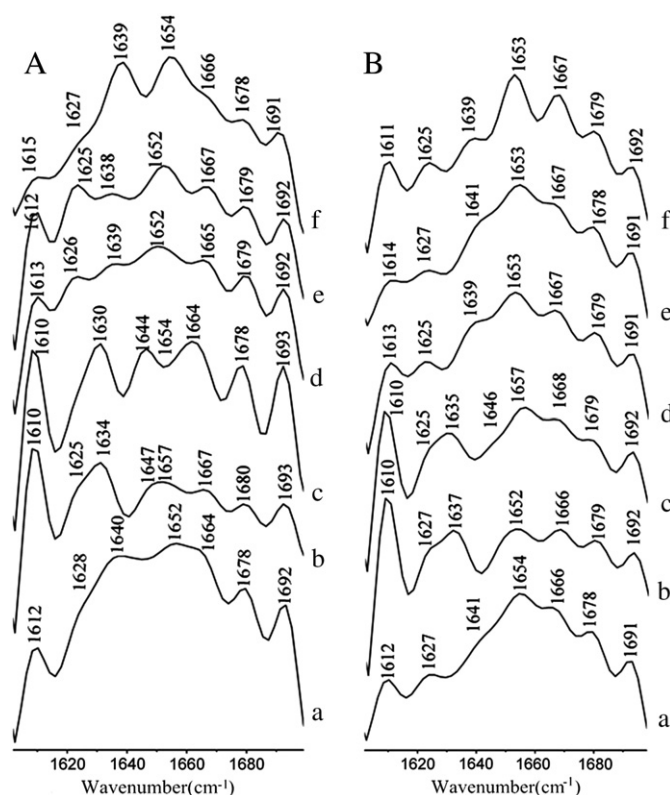


Fig. 5. Stacked plot of deconvoluted infrared spectra of OVA (A)/LYZ (B) without/with TP. (A): (a) OVA (pH1.2), (b) OVA/TP = 1:0.2 (pH1.2), (c) OVA/TP = 1:1 (pH1.2), (d) OVA (pH7.5), (e) OVA/TP = 1:0.2 (pH7.5), and (f) OVA/TP = 1:1 (pH7.5); (B): (a)–(f) are similar to (A) except that it is LYZ instead of OVA.

amide I band at 1660 cm^{-1} (mainly C=O stretch) and amide II band at 1530 cm^{-1} (C–N stretching coupled with N–H bending modes) (Krimm, 1983) upon polyphenol interaction. When the ratio of polyphenol and OVA/LYZ is 1:1, blue shifts are observed in the different spectra for amide II bands at $\sim 1530\text{ cm}^{-1}$. Compared with free protein spectra, the degrees of blue shifts of TP-OVA (1.2), TP-OVA (7.5), TP-LYZ (1.2), and TP-LYZ (7.5) are -18 cm^{-1} , -10 cm^{-1} , -18 cm^{-1} , and -7 cm^{-1} , respectively (Fig. 4). Spectral shifts observed at 1660 cm^{-1} and 1530 cm^{-1} indicate that there is a conformational change of OVA/LYZ affected by TP (Papadopoulou et al., 2005). Degree of blue shifts at pH 1.2 is much greater than that of pH 7.5 on both proteins with the addition of TP, which suggested that a stronger conformational change occurred at pH 1.2 compared with that at pH 7.5 of both proteins. This result agreed with the results of Fluorescence experiments. Two new peaks of OVA and LYZ at pH 1.2, 1632 cm^{-1} (β -sheet) and 1637 cm^{-1} (3_{10} -helix or β -sheet) respectively (Byler & Susi, 1986; Choi & Ma, 2005), were observed for the protein–polyphenol

mixture (protein:TP = 1:1), as compared with homogenous protein spectra at pH 1.2 (Fig. 4). It suggested that the structure of OVA/LYZ tend to with more loosely packed β -sheet structures at pH 1.2 affected by TP (Kauffmann, Darnton, Austin, Batt, & Gerwert, 2001). However, no peaks at 1632 cm^{-1} and 1637 cm^{-1} could be found at pH 7.5 of both proteins.

Fig. 5 showed the stacked deconvoluted infrared amide I spectrum ($1600\text{--}1700\text{ cm}^{-1}$) of OVA/LYZ with and without TP. Seven major bands associated with conformation of proteins were distinctly observed in the amide I region of these deconvoluted curves. The wavenumbers of these characteristic fitted-peaks of free OVA/LYZ are similar to the results of Byler & Susi (Byler & Susi, 1986). Based on literatures, these major bands in amide I region of OVA/LYZ could be assigned as follows: The bands located at 1612 and 1625 cm^{-1} are due to β -sheet structures. The band at 1654 cm^{-1} corresponds to the α -helix structure. The band is located at 1638 cm^{-1} due to 3_{10} -helix or β -sheet structure. The bands at 1645 cm^{-1} and 1691 cm^{-1} are assigned to random coil structure and β -type structure, respectively. The bands around 1667 and 1678 cm^{-1} are generated from β -turn structures (Byler & Susi, 1986; Choi & Ma, 2005). A quantitative analysis of the protein secondary structure for the free OVA/LYZ and its polyphenol adducts had been carried out and the results were shown in Tables 3A and 3B. Secondary structures of both proteins affected by polyphenol are similar. At pH 1.2, TP increased β -sheet structures in both proteins at the expense of α -helix; for OVA β -sheet structures increased from 18 to 31% and for LYZ from 14 to 25%; α -helix structures decreased from 18 to 12% for OVA and 22 to 16% for LYZ (Tables 3A and 3B). New peaks of 1644 cm^{-1} (random) for OVA and 1646 cm^{-1} (random) for LYZ increased with the addition of TP (LYZ/TP = 1:1) at pH 1.2 (Fig. 5A and B), which suggested an increase of irregular structure. At pH 7.5, the result of secondary structures of both proteins affected by polyphenol contrasted from that of pH 1.2. Tables 3A and 3B showed that random and β -sheet structures decreased and α -helix structures increased of both proteins with the addition of TP at pH 7.5.

4. Discussion

The results of our digestion experiments showed that TP made OVA/LYZ easier for digestion in the pepsin solution at pH 1.2 and inhibited OVA/LYZ digestion in pancreatin solution at pH 7.5. These results are consistent with gastric digestion studies of food allergens (β -lactoglobulin, α -lactalbumin and peanut allergens) in the presence of catechin-enriched polyphenol extract of green tea previously reported (Tantoush et al., 2012). To explore the impact mechanism of TP on protein digestion, many researchers have made a lot of efforts. Sangkil Nam et al. reported that ester bond-containing tea polyphenols potently inhibited proteasome activity in vitro and in vivo. Qiang He et al. concluded that the enzyme activity inhibition ratios of α -amylase, pepsin, trypsin and lipase were, respectively, 61%, 32%, 38% and 54% in the presence of 0.05 mg/ml tea polyphenols. And they attributed the inhibiting effect to the main mechanism of TP-enzyme binding. However, our

Table 3A

Influence of TP binding on the OVA secondary structure at pH 1.2 and 7.5. Percentage of secondary structure motifs are calculated by Peakfit TM software. The ratios of protein and TP are 1:0.2 and 1:1.

Assignment	pH1.2 (%)			pH7.5 (%)		
	OVA	OVA-TP (1:0.2)	OVA-TP (1:1)	OVA	OVA-TP (1:0.2)	OVA-TP (1:1)
β -sheet	18.2 $\pm$ 0.6a	29.4 $\pm$ 0.3b	30.8 $\pm$ 0.7b	23.9 $\pm$ 0.2b	27.2 $\pm$ 0.3c	12.3 $\pm$ 0.3a
3_{10} -helix or β -sheet	17.2 $\pm$ 0.2c	15.4 $\pm$ 0.2b		15.9 $\pm$ 0.2a	15.3 $\pm$ 0.4a	21.4 $\pm$ 0.5b
α -helix	18.1 $\pm$ 0.3c	14.4 $\pm$ 0.4b	12.4 $\pm$ 0.2a	19.0 $\pm$ 0.2a	19.9 $\pm$ 0.2b	22.3 $\pm$ 0.4c
β -turn	33.9 $\pm$ 0.5c	21.0 $\pm$ 0.8a	31.1 $\pm$ 0.3b	30.8 $\pm$ 0.2b	28.4 $\pm$ 0.2a	31.0 $\pm$ 0.3b
β -type	12.6 $\pm$ 0.1b	7.7 $\pm$ 0.3a	11.9 $\pm$ 0.4b	10.4 $\pm$ 0.4b	9.2 $\pm$ 0.0a	13.0 $\pm$ 0.0c
Random		12.2 $\pm$ 0.9a	13.8 $\pm$ 1.4b			

Values are expressed as % $\pm$ standard error. All values within half a row with different letters are significantly different, $p < 0.05$

Table 3B

Influence of TP binding on the LYZ secondary structure at pH 1.2 and 7.5. Percentage of secondary structure motifs are calculated by Peakfit TM software. The ratios of protein and TP are 1:0.2 and 1:1.

Assignment	pH1.2 (%)			pH7.5 (%)		
	LYZ	LYZ-TP (1:0.2)	LYZ-TP (1:1)	LYZ	LYZ-TP (1:0.2)	LYZ-TP (1:1)
β-sheet	14.2 ± 1.0a	32.0 ± 0.8c	24.8 ± 0.8b	17.2 ± 0.3b	12.1 ± 0.9a	16.9 ± 0.5b
α-helix	21.8 ± 0.4c	17.5 ± 0.3b	16.1 ± 0.2a	21.3 ± 0.4a	22.4 ± 0.2a	24.2 ± 0.7b
β-turn	37.1 ± 0.8b	29.3 ± 0.6a	26.8 ± 1.4a	33.3 ± 0.8a	36.6 ± 0.5b	35.6 ± 0.1b
β-type	12.5 ± 0.6b	9.9 ± 0.2a	9.7 ± 0.4a	11.7 ± 0.7b	12.7 ± 0.1c	9.6 ± 0.3a
Random	14.5 ± 0.4b	11.3 ± 1.4a	22.6 ± 1.2c	16.5 ± 0.3b	16.3 ± 0.6b	13.8 ± 0.1a

Values are expressed as % ± standard error. All values within half a row with different letters are significantly different, $p < 0.05$

results showed that TP improved the enzymatic rate of the pepsin. This was an interesting phenomenon that enzymolysis rate was higher while enzyme activity was reduced. This phenomenon might be due to the structural transformation of substrate affected by TP, which was easier to be digested by pepsin. Therefore, we suggested that TP-protein (TP-substrate) was another mechanism of TP affecting digestion.

Fluorescence quenching analysis showed that interaction happened between TP and proteins at pH 1.2 and pH 7.5. Results of both Fluorescence and FTIR indicated that greater conformational change occurred to OVA/LYZ at pH 1.2 affected by TP, compared with that of pH 7.5. This result might due to the solution conditions. Acidic conditions could promote structural unfolding and conformational change of bovine serum albumin (Estey, Kang, Schwendeman, & Carpenter, 2006). Therefore, acidic conditions might induce the protein conformation to a much looser conformation, and TP might reinforce this effect. OVA is a monomer phosphorus glycol-protein which contained three tryptophan residues: Trp148 and Trp 267 in helical region, and Trp184 in beta-strand region. The Trp148, Trp267, and Trp184 residues are the most dominant fluorophores and played an important role in the quaternary state change upon ligand binding (Wang et al., 2013). LYZ molecule has six tryptophan residues, the tertiary structure was comprised of two folding domains, one domain is formed from four α-helices (α-domain), and the other domain is formed from the triple-stranded β-sheet and the 3_{10} -helix (β-domain) (Yokota, Izutani, Takai, Kubo, Noda, Koumoto, et al., 2000). The active site is formed at the interface between two domains, and it includes three tryptophan residues (Trp62, 63 and 108). The other three (Trp28, 111 and 123) were included in the hydrophobic core region in the α-domain (Cowgill, 1967; Zakin & Herschbach, 1986). Results of Fluorescence quenching showed that the microenvironment of tyrosine and tryptophan residues was changed and became hydrophobic, which suggested that second structures and active sites of OVA/LYZ might change. More unfolded and looser conformation of OVA/LYZ might expose more enzyme target sites for pepsin affected by acidic conditions and TP at pH 1.2, thus making OVA/LYZ easier to be digested by pepsin.

At pH 1.2, the FTIR results exhibited that random structures and β-sheet structures increased and α-helix decreased in both proteins affected by TP. Akama et al. and Kinsella et al. have reported that α-helix is a very stable structure due to the fact that the proximal end of the cavity is tightly sealed, thereby not permitting the entry of any molecule, while β-sheet and random structures own looser structure compared to α-helix (Akama, Kanemaki, Yoshimura, Tsukihara, Kashiwagi, Yoneyama, et al., 2004; Kinsella, 1982). According to these reports, we suggested the structure of OVA/LYZ might be much looser affected by TP at pH 1.2. These proteins with looser second structure might be easier to digest. Opposite structural changes of both proteins affected by TP happened at pH 7.5. The increase of α-helix suggested that the second structure of OVA/LYZ become more compact at pH 7.5 affected by TP. The decrease of random structures of OVA/LYZ with the addition of TP showed the loss of irregular structures, which might result in much more compact structures of proteins (Kinsella, 1982).

In conclusion, TP-protein (TP-substrate) was also a mechanism of TP affecting digestion. Different degrees of conformational change occurred to OVA/LYZ at pH 1.2 and pH 7.5 affected by TP, which might lead to different degrees of unfolding of OVA/LYZ and affected the digestibility. Different changes of second structure of proteins (substrate) affected by TP at pH 1.2 and pH 7.5 might also result in the different digestibilities between pH 1.2 and pH 7.5.

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