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Low pH-shifting treatment would improve functional properties of black turtle bean (*Phaseolus vulgaris* L.) protein isolate with immunoreactivity reduction

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ABSTRACT

Low pH-shifting was firstly applied in the black turtle bean (*Phaseolus vulgaris* L.) protein isolate treatment by acidic (pH 1.0–3.0) buffer incubation for 8 h, then was adjusted to pH 7.2 and kept 3 h for protein stabilizing. Mild loss of secondary structure was confirmed in the protein isolate after low pH-shifting treatment by CD and FT-IR analyses. Intrinsic fluorescence, UV spectra, surface hydrophobicity, SH content and SDS-PAGE analyses indicated the protein conformation was unfolded with the exposure of much more buried hydrophobic residues, which would result in the enhancement of emulsifying properties, foaming properties and fat holding capacity, and lead to the reduction of solubility and water holding capacity. Furthermore, lower immunoreactivity was observed by the ELISA, and improved digestibility was found in *in vitro* digestion assay. Our results suggested the low pH-shifting treatments would broaden the application of bean protein isolate with better hydrophobic processing functions and safety.

1. Introduction

Phaseolus vulgaris beans, also known as common beans, are among the most important varieties of cultivated grain legumes in the word, which have been served as the health-enhancing and better functional compositions in several dishes for direct human consumption, such as salads, casseroles, macaroni, and Rajma-chawal (He et al., 2018; Kumar, Verma, Das, Jain, & Dwivedi, 2013). However, not only the functionalities of *P. vulgaris* bean protein isolate, such as surface-active properties (e.g. emulsifying property), inherent protein digestibility and other processing properties, were limited in the food manufacture (Carrasco-Castilla et al., 2012; Tang, 2008; Yin, Tang, Wen, & Yang, 2010), but the higher allergenic potential of the protein isolate also would restrict the wide applied due to the rich lectin content (He et al., 2018; Kumar et al., 2013).

Therefore, several processing technologies, such as high pressure (Yin, Tang, Wen, Yang, & Li, 2008), ultrasonic (Fan et al., 2014), limited enzymatic hydrolysis (López-Barrios, Antunes-Ricardo, & Gutiérrez-Uribe, 2016) as well as PEGylation (He et al., 2018; Yang et al., 2018), have been utilized for the structural modifications of the *P. vulgaris* bean protein isolate in order to obtain the enhanced processing functionalities and digestibility (Yin et al., 2008) and/or lower allergenicity (He et al., 2018; Yang et al., 2018). However, these methods were significantly hampered in the industrial applications by the additional cost, tedious operations and potential safety risks, and could not be adopted in the near future (Ekezie, Cheng, & Sun, 2018). To our knowledge, to date, fewer mild and economical technique has been established to improve processing properties of *P. vulgaris* bean protein isolate with decreasing allergenicity.

As an inevitable strategy in food processing, pH adjustment is always the basic primary process in the protein preparation. Recently, more and more protein researchers were concerning about the conformational changes of the food protein molecules by adjusting the pH to neutrality after a low acidic condition treatment (i.e., pH-shifting) (Kristinsson & Hultin, 2003a, 2003b; Jiang, Chen, & Xiong, 2009; Jiang & Ding, 2017). Emulsifying properties and gel strength of cod myosin were raised after a pH 2.5 shifting treatment (Kristinsson & Hultin, 2003a, 2003b), and recent studies suggested that emulsifying

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properties and gelling properties of soy protein isolate (SPI) were markedly improved by the pH 1.5 shifting treatment and/or comb with mild heating (Jiang et al., 2009; Liu, Geng, Zhao, Chen, & Kong, 2015). Furthermore, considering the industrial protein isolate production process of "alkali solution acid precipitation", the low pH induced treatment on the allergenicity and *in vitro* digestibility of lectin protein from black turtle bean (*P. vulgaris* L.) has been explored in our previous study, and the tertiary structure of the protein was proven to be unfolded with the reduction of IgE binding capacity (Zhao, He, Tang, Sun, Zhang, Ye, et al., 2019). However, detailed information of low pH-shifting treatment on the structure and functionality alterations of black turtle bean protein isolate have not been well documented yet.

Thus, in the present study, low pH-shifting treatment was applied in black turtle bean (*P. vulgaris* L.) protein isolate processing. Structural alterations of the protein isolates were explored by circular dichroism (CD) spectra, Fourier transform infrared (FTIR) spectra, intrinsic fluorescence spectra, UV absorption spectra, surface hydrophobicity, sulfhydryl (SH) contents and SDS–PAGE analysis, respectively. The effects of low pH-shifting on solubility, gelling properties, emulsifying and foaming properties, fat-holding and water capacities, hemagglutination activity, immunoreactivity and *in vitro* digestibility were further investigated. Our findings would give an insight into the relationship between structural alterations and functional properties for *P. vulgaris* L. bean protein isolate, which would be beneficial to the tremendous application of the protein isolate in the developments of nutrition and safe legume based foods.

2. Materials and methods

2.1. Black turtle beans protein isolate preparation

Commercial dried black turtle beans (*P. vulgaris* L.) cultivated in Heilongjiang Province of China were purchased from a local market (Hefei, Anhui, China). Black turtle beans protein isolate was prepared as the method described by Yang et al. (2018) with minor modifications. Black turtle beans were first milled to pass through 80-mesh sieve, and were treated with petroleum ether (1:10, w/v) for degreasing. Them the defatted bean meal was suspended in 10-fold weight of deionized water, and was adjusted to pH 7.2 with 2 M NaOH to extract protein at 25 °C for 12 h in a stirring state. After centrifugation at 8000 rpm for 30 min, the supernatants were adjusted to pH 4.5 with 2 M HCl, and the precipitates were obtained by using centrifugation at 8000 rpm for 30 min, then the proteins were recovered in the 10-fold weight of deionized water, and were dialyzed against deionized water for 48 h at 4 °C. Protein isolate powder was obtained by the freeze-dried, and was stored at − 80 °C until use. Protein content in the prepared black turtle beans protein isolate powder was 85 ± 1% (w/w) as determined by using the Kjeldahl method (N × 5.8) (AOAC, 2000; method 976.05).

2.2. Low pH-shifting treatment

Black turtle bean protein isolate was firstly subjected to low pH incubation treatment according to our previous method (Zhao et al., 2019), as the protein isolate (2.0 mg/mL) was dissolved in a specific pH buffer (pH 3.0, 2.0, 1.5 and 1.0, respectively) and was maintaining for 8 h at room temperature (25 ± 2 °C). Then, pH-shifting process was carried out to neutralize the protein solution to pH 7.2 by using 2 M NaOH, and held for 3 h for the stability. The protein isolate powder was obtained by the freeze-drying after a dialysis, and the untreated protein sample was set as the native control.

2.3. Circular dichroism (CD) spectra analysis

CD spectra data between 190 nm and 260 nm were collected by using a Chirascan™ quantitative circular dichroism spectrometer (Applied Photophysics Ltd., London, UK) at 25 ± 2 °C. The spectra were recorded at a 90 nm/min scanning speed in a 1 mm path length quartz cuvette under nitrogen atmosphere. Each protein sample (0.2 mg/mL) was prepared in the phosphate buffer (10 mM, pH 7.2), and scanned 3 times to obtain an averaged value.

2.4. Fourier transform infrared spectroscopy (FT-IR) analysis

The lyophilized protein sample (5.0 mg) was mixed with KBr (100 mg) and ground into a fine powder in an agate mortar incubated with infrared light, and then was pressed into a pellet. A Nicolet 67 FT-IR spectrometer (Thermo Nicolet Co., USA) was employed to obtain the FT-IR spectra data in the wave number range from 400 to 4000 cm^{-1} during 32 scans with 0.09 cm^{-1}. The results were further analyzed by Omnic 8.2 software (Thermo Fisher Scientific Inc., Madison, WI) and Peakfit 4.12 (Systat Software Inc., San Jose, CA).

2.5. Intrinsic fluorescence measurement

Fluorescence measurements were performed on a RF-20AXS spectrofluorometer (Horiba Ltd., Kyoto, Japan). Each sample (0.2 mg/mL) was prepared in 10 mM phosphate buffer at pH 7.2. Intrinsic fluorescence emission between 300 and 400 nm was recorded at a 280 nm excitation with a slit width of 5 nm, and all spectra were corrected by subtraction of phosphate buffer.

2.6. UV absorption spectra measurements

The UV spectra of protein samples (0.5 mg/mL) were recorded from 240 to 320 nm with a wavelength interval at 1.0 nm by using an UV–vis spectrophotometer (UV-4802, Unico Instrument Co., Ltd. USA) at room temperature (25 ± 2 °C) with the corresponding buffer as the blank. The second-derivative UV spectra were further analyzed by using Origin 8.5 software (Origin-Lab Co., Northampton, USA).

2.7. Surface hydrophobicity analysis

The surface hydrophobicity (H_0) of the protein sample was analyzed according to the method of Liu et al. (2015). Briefly, four milliliters aliquots of the treated samples (0.001–0.6 mg/mL) were prepared in 10 mM phosphate buffer at pH 7.2, respectively, and mixed with 50 μL of 8 mM ANS solution for a 5 min reaction. Then, the fluorescence intensity was determined with an emission at 480 nm at a 380 nm excitation on a RF-20AXS spectrofluorometer (Horiba Ltd., Kyoto, Japan) both with a slit width of 5 nm. The plot of fluorescence intensity versus protein concentration was employed to measure the protein H_0.

2.8. Total sulfhydryl (SH) and exposed SH contents measurements

Protein sample (15 mg) was dissolved in the 5.0 mL reaction buffer (90 mM Gly, 86 mM Tris, and 4 mM EDTA, pH 8.0) with (total SH) or without (exposed SH) 8 M urea, and fifty microliters of Ellman's reagent was following added. After a 60 min incubation at room temperature (25 ± 2 °C), a centrifugation of 8000 rpm for 15 was applied, and the absorbance of supernatant was recorded at 412 nm with the reagent buffer as blank control. The total SH and exposed SH contents in the protein sample (mg/mL) were calculated by using the extinction coefficient of NTB (13,600 M^{-1} cm^{-1}).

2.9. Electrophoresis analysis

The protein sample (2.0 mg/mL) was boiled in loading buffer with and without β-mercaptoethanol at 1:1 (v/v) ratio for 5 min, and was analyzed by gel electrophoresis according to the previous method of Liu and Xiong (2000). A stacking gel with 4% acrylamide and a gradient resolving gel with 12% acrylamide were employed. For the SDS–PAGE

sample without β-mercaptoethanol, 1 mM *N*-ethylmaleimide (NEM) was added to prevent the formation of disulfide artifacts. Then, 10 μL of each sample was loaded in each well of the SDS–PAGE gel.

2.10. Colour determination

The colour of protein isolate powder was performed on a Chroma Meter (Shenzhen 3NH Technology Co., Ltd., Shenzhen, China), and the value of L^* (brightness or luminosity), a^* (redness → greenness) and b^* (blueness → yellowness) was recorded, respectively.

2.11. Solubility determination

Each protein sample (1.0%, w/v) was prepared in 10 mM phosphate buffer at pH 7.2, and completely dissolved sample was obtained by using the 0.2 M NaOH as control. After the centrifugation at 10000 rpm for 30 min, the protein content in the supernatant was determined by the Brodford method by using the bovine serum albumin as the standard curve (0–100 μg/mL). Protein solubility (PS) was calculated according to Eq. (1).

$$PS(\%) = \frac{C_1}{C_0} \times 100\% \quad (1)$$

where C_1 was the protein content of sample (mg/mL), C_0 was the total soluble protein content (control, mg mL^{-1}).

2.12. Gel strength testing

The gel strength of protein sample was determined following the report of Liu et al. (2015). Protein sample (12%, w/v) was prepared individually by phosphate buffer (10 mM, pH 7.2) in a 50 mL beaker, and incubated in a 90 °C water bath for 30 min, then the sample was rapidly cooled to 25 ± 2 °C in the ice slurry and stored in a 4 °C refrigerator overnight. All of the samples were prepared in triplicate. Gel strength was determined by using a TA-XTplus texture analyzer (Stable Micro Systems Ltd., Godalming, Surrey, U.K.) with a cylinder measuring probe (P/0.5, 12 mm in diameter), and the gel strength was defined as the force required to rupture the gel.

2.13. Emulsifying properties measurements

Turbidity measurements were applied to determine the emulsifying properties (Pearce & Kinsella, 1978). Each protein sample (1.0 mg/mL) was dispersed in 10 mM phosphate buffer at pH 7.2. Thirty milliliters of treated samples were mixed with 5 mL of squeezing soybean oil, and the mixture was homogenized by an IKA T18 ultra-turrax (Werke GmbH & Co., Staufen, Germany) at a speed of 22,000 rpm for 3 min. Then an aliquot of the emulsion (50 μL) was pipetted from the bottom of the container at 0 and 10 min after homogenization, and was mixed with 5 mL of 0.1% sodium dodecyl sulphate (SDS) solution. The absorbance of the diluted solution was measured at 500 nm by using an UV–vis spectrophotometer (UV-4802, Unico Instrument Co., Ltd. USA). Emulsifying activity index (EAI) and emulsion stability index (ESI) were calculated by the following Eq. (2) and (3):

$$EAI(m^2/g) = \frac{2 \times 2.303 \times A_0}{[0.25 \times \text{protein weight(g)}]} \quad (2)$$

$$ESI(\text{min}) = \frac{A_0 \times \Delta t}{\Delta A} \quad (3)$$

where A_0, A_{10} represented the absorbance at 0, 10 min after homogenization, respectively; $\Delta t = 10$ min; and $\Delta A = A_0 - A_{10}$.

2.14. Foaming properties measurements

Foaming capacity and stability were measured according to the method of Deng et al. (2011). Ten milliliters of protein sample solutions (1.0% protein concentration, w/v, in 10 mM phosphate buffer at pH 7.2) were prepared in the 50 mL graduated centrifuge tube with Volume calibration. The air was then dispersed in the protein solution by using a high speed homogenizer (Werke GmbH & Co., Staufen, Germany) at the speed of 12,000 rpm for 2 min. Foaming capacity (FC) and foaming stability (FS) were evaluated by using Eqs. (4) and (5), respectively.

$$FC\ (\%) = \frac{V_0 - V}{V} \times 100\% \quad (4)$$

$$FS\ (\%) = \frac{V_0 - V_{10}}{V_0 - V} \times 100\% \quad (5)$$

where V was the initial volume, V_0 was the volume immediately after foaming and V_{10} was the volume of liquid remaining after 10 min at room temperature.

2.15. Fat holding capacity determination

Fat holding capacity (FHC) was analyzed by using the method of Deng et al. (2011). The protein isolate (0.5 g) was weighed into a 10 mL pre-weighed centrifuge tube, and was thoroughly mixed with 3 mL of soybean oil by using a vortex mixer for 5 min. After centrifugation at 5000 g for 20 min, the supernatant was carefully discarded, and the tubes and precipitate were re-weighed. Then, FHC was calculated as Eq. (6),

$$FHC\ (g/g) = \frac{F_2\text{-}F_1}{F_0} \quad (6)$$

where F_0 was the weight of the sample (g), F_1 was the weight of the tube plus the sample (g), and F_2 was the weight of the tube plus the precipitate (g).

2.16. Water holding capacity measurements

The water holding capacity (WHC) was measured based on the method of He et al. (2018). The lyophilized protein powder was vigorously mixed with distilled water to a final protein concentration of 50 mg/mL for 5 min. After 60 min equilibrium, the suspension was centrifuged at 5000 rpm for 20 min at 25 °C, then the supernatant was decanted and the precipitate was dried by a vacuum freeze dryer. The WHC was calculated as Eq. (7),

$$WBC\ (g/g) = \frac{W_1\text{-}W_2}{W_0} \quad (7)$$

where W_0 was the weight of the protein sample (g), W_1 was the weight of the tube plus the precipitate (g), and W_2 was the weight of the tube plus the dry sample (g).

2.17. Hemagglutination activity assay

Hemagglutination activity was determined based our previous method (He et al., 2018). The serial twofold dilutions of the protein samples were performed by using the erythrocyte buffer (75 mM PBS-NaCL buffer, pH 7.2) in a 96 well V shape microliter plate at the room temperature, then the same volume of 2% (w/v) rabbit blood cell suspension was added into each well for a 2 h incubation at 4 °C. The hemagglutination activity (HA, HU/mg) of protein sample was represented as the unit of activity (2^n) per mg of protein, and the n was the highest dilution ratio showing the detectable hemagglutination.

2.18. Immunoreactivity determination

The immunoreactivity of the protein sample was measured by ELISA according to our previous method (Zhao et al., 2019). Protein sample

(5 μg/mL, 100 μL per well) in Na_2CO_3 buffer (50 mM, pH 9.6) was coated in the ELISA plate, and was incubated overnight at 4 °C. After washing with phosphate buffer (pH 7.2, 10 mM) twice, the plate was blocked by 5% (w/v) defatted milk (200 μL per well) at 25 °C for 1 h. Then, the plate was washed and incubated with mouse anti-lectin serum (1:1000 v/v dilution with PBS pH 7.2, 10 mM) obtained from Nanjing SenBeiJia Biological Technology Co. Ltd. (Nanjing, Jiangsu Province, China) as the primary antibody via subcutaneous injection sensibilization of the purified lectin to the mouse. After overnight incubation at 4 °C, the plate was following washed and incubated with 1:1000 (v/v) diluted HRP labeled goat anti-mouse IgE antibody (Sigma-Aldrich Chemicals Co., St. Louis, MO, USA) at 37 °C for 2 h. Finally, the chromogenic reaction was developed by using 3,3′, 5,5′-tetramethylbenzidine (TMB) for 10 min in the dark and was terminated with 0.2 M sulphuric acid. The optical density (OD value) was determined at 450 nm within 15 min by using a Tecan Infinite™ 200Pro microplate reader (Tecan Group Ltd., Mannerdorf, Switzerland).

2.19. In vitro digestion assay

The protein samples were subjected to *in vitro* simulated gastrointestinal digestion by the hydrolysis of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) in sequence according to our previously study (Zhao et al., 2019). Briefly, a porcine pepsin: protein samples ratio of 1:20 (w/w) was applied in the SGF digestion process, and aliquots (100 μL) of samples were taken at 0, 10, 20, 30 and 60 min, respectively. The SGF digestion reaction was terminated by adding 0.2 M Na_2CO_3 solution to adjust the pH to more than 9.0, and then the pH of digestion solution was adjusted to pH 7.2 with 1.0 M NaOH solution for the SIF digestion at the ratio of trypsin to protein was 1:100 (w/w). The digesting reaction was stopped by a boiling heating for 10 min, and the 100 μL of the SIF reaction solution was taken as 5, 10, 30 and 60 min, respectively. All the digestion samples were analyzed by using SDS-PAGE.

2.20. Statistical analyses

All experiments were repeated at least of triplicates and the values were analyzed as mean ± standard deviation. Analyses of variance (ANOVA) and Duncan's means comparison test were applied with a significance level of 0.05by using SPSS software (version 11.5, SPSS Inc. Chicago, IL, USA).

3. Results and discussion

3.1. Structural analysis

3.1.1. CD spectra

As shown in Fig. 1a, two evident negative bands at around 208 and 222 nm were found in the CD curves, which are typical characteristic of the proteinα-helix, and a negative peak at around 216 nm attributed to β-sheet contents (Greenfield & Fasman, 1969). With respect to native control, evident decreases in negative CD signal value at 208 and 222 nm were observed in the low pH-shifting protein samples, and the prominent shift of the peak wavelength, both from 222 and 208 nm to 216 nm, indicated the decreasing in α-helix content of black turtle beans protein isolate accompanying with an increasing in β-sheet structure induced by the pH-shifting treatment in the acidic pH sequence, and the phenomena suggested that the secondary structure contents would be affected in the low pH incubation, especially for the pH 1.5 and 1.0 treatments, and it should be noted that the conformation might not be completely revised in the protein refolding process. Similar structural changes were observed in soy protein isolates subjected to pH-shifting treatment from pH 1.5 (Jiang et al., 2009; Liu et al., 2015).

3.1.2. FT-IR spectra

The conformational changes of the low pH-shifting protein isolates were further investigated by the FI-IR analysis, as the curve-fitted spectra of the amide I band (1600–1700 cm^{-1}) in FT-IR were highly sensitive to the alterations of protein secondary structures, and 1610–1638 cm^{-1} belonged to β-sheet, 1638–1648 cm^{-1} attributed to random coil, 1649–1660 cm^{-1} belonged to α-helix, and 1660–1692 cm^{-1} attributed to β-turn structures (He et al., 2015). As shown in Fig. 1b-f, compared to the native control, the α-helix contents decreased from 20.82% to 15.55% after the pH-shifting with the decreasing of incubation pH values, while the β-sheet contents increased from 33.08% to 42.49%. These data were in good agreement with the results of the CD analysis, and suggested that a misfolding of protein conformation might have happened after the treatments. According to the previous studies, the hydrogen bond would play a crucial role in formation and stabilization of helical structure, and the amino acid groups (–OH, –COOH and $–NH_2$) on protein molecules would be protonated when the protein was incubated in the environmental pH below the isoelectric point, and the protein would be unfolded because of the increase of protein intramolecular electrostatic repulsions, then the structures might be rearranged in the refolding process when the pH was adjusted to the neutral (Goto, Calciano, & Fink, 1990; Kristinsson & Hultin, 2003a, 2003b). Besides, the loss of the helical structure indicated the flexibility enhancement of the pH-shifting protein isolate, especially for the pH 1.0 and 1.5 treated protein samples, and the results could be speculated that the protein hydrophobicity raised (Liu et al., 2015).

3.1.3. Intrinsic fluorescence

Structural changes in the tertiary structure of black turtle bean protein isolate were investigated by intrinsic fluorescence spectroscopy (Fig. 2a). Since the florescence emission of Tyr redidues might be quenched because of the interaction with the molecular chain or energy transfer to Trp residues, only the Trp residues could be detectable in most globular protein fluorescence spectra excited at 280 nm, then the maximum fluorescence emission wavelength (λ_{max}) of the untreated protein isolate (control) was found at around 330 nm, confirming the hydrophobic environment of the Trp residues in the protein isolate (Zhao et al., 2019), which was similar to that of red kidney bean (*P. vulgaris* L.) protein isolate (Yin et al., 2008). In our previous study for the black turtle bean lectin (Zhao et al., 2019), the tertiary structure of the protein was unfolding during the first 8 h low pH incubation showing the blue shifting and fluorescence intensity decreasing. As shown in Fig. 2a, decreases in fluorescence intensity of the black turtle bean protein isolate were obviously observed along with mildly red shifts (1–2 nm) in the λ_{max} with the decrease of pH value that the protein sample would be shifted from, suggesting the conformational difference between the low pH-shifting proteins after refolding and native control. The fluorescent phenomena might be attributed to the exposure of Trp residues to polar environments compared to the native protein state, and the results suggested the rearrangement of the protein conformation with the exposure of hydrophobic residues (Kristinsson & Hultin, 2003a, 2003b; Zhao et al., 2019).

3.1.4. UV spectra

The second-derivative UV spectroscopy was applied to monitor the changes of tertiary structure for protein molecules (He et al., 2015). As shown in Fig. 2b, the peak-to-trough values near 260, 285 and 295 nm were attributed to the absorbance of Phe, Tyr and Trp residues, respectively, which would reflect the environments of the aromatic amino acid residues (Wang, Yang, Yin, Zhang, Tang, Li, et al., 2011). A slight blue-shift of peak in the Tyr residue region (near 285 nm) was found with the decreasing of original pH value from 3.0 to 1.0, indicated that partial protein unfolding or structural rearrangement was still exited in the low pH-shifting samples with the exposure of more Tyr residues to hydrophilic region (Wang et al., 2011). Furthermore, since the

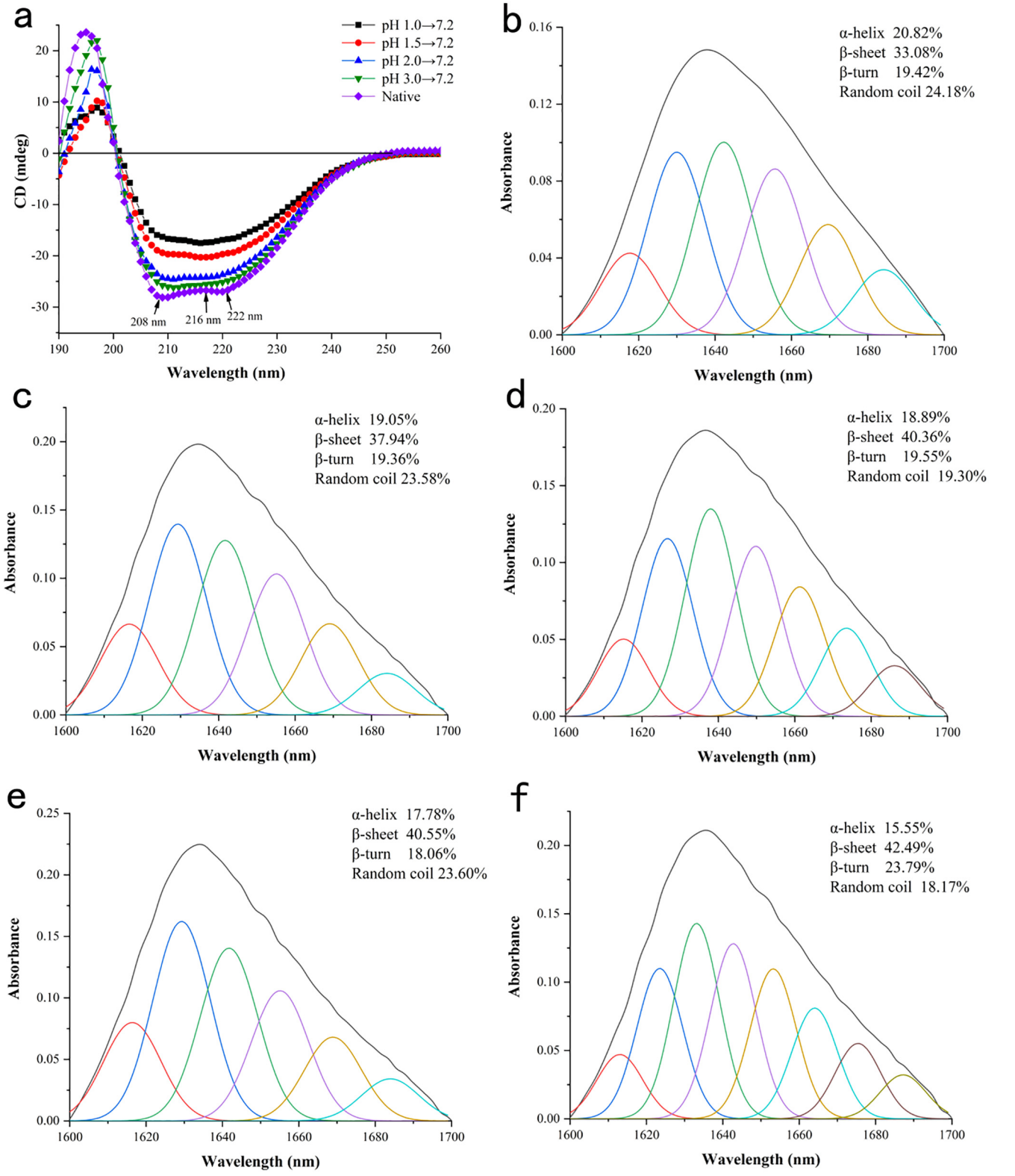

Fig. 1. Effects of low pH-shifting treatment on secondary structure of black turtle bean (*P. vulgaris* L.) protein isolates. (a) Changes in CD spectra. FT-IR spectra and the curve fitting results of amide I (1600–1700 cm^{-1}) of native control (b) and low pH-shifting (c, pH 3.0 → 7.2; d, pH 2.0 → 7.2; e, pH 1.5 → 7.2 and f, pH 1.0 → 7.2) samples.

amplitude of the derivative spectral bands, described by calculating the ratio (r = a/b) of the two peak-to-trough values, was also sensitive to the Tyr residue changes, a gradual increase in r values from 1.389 (native) to 1.948 (pH 1.5 → 7.2) was found after the low pH-shifting process (Fig. 2b), confirmed that more Tyr residues moved to the polar microenvironments, and the r value reduction of pH-shifting sample from 1.0 to 7.2 suggested the slight conformational alterations after the refolding as the r value had been well related to the Tyr/Trp ratio in the proteins (Lange & Balny, 2002).

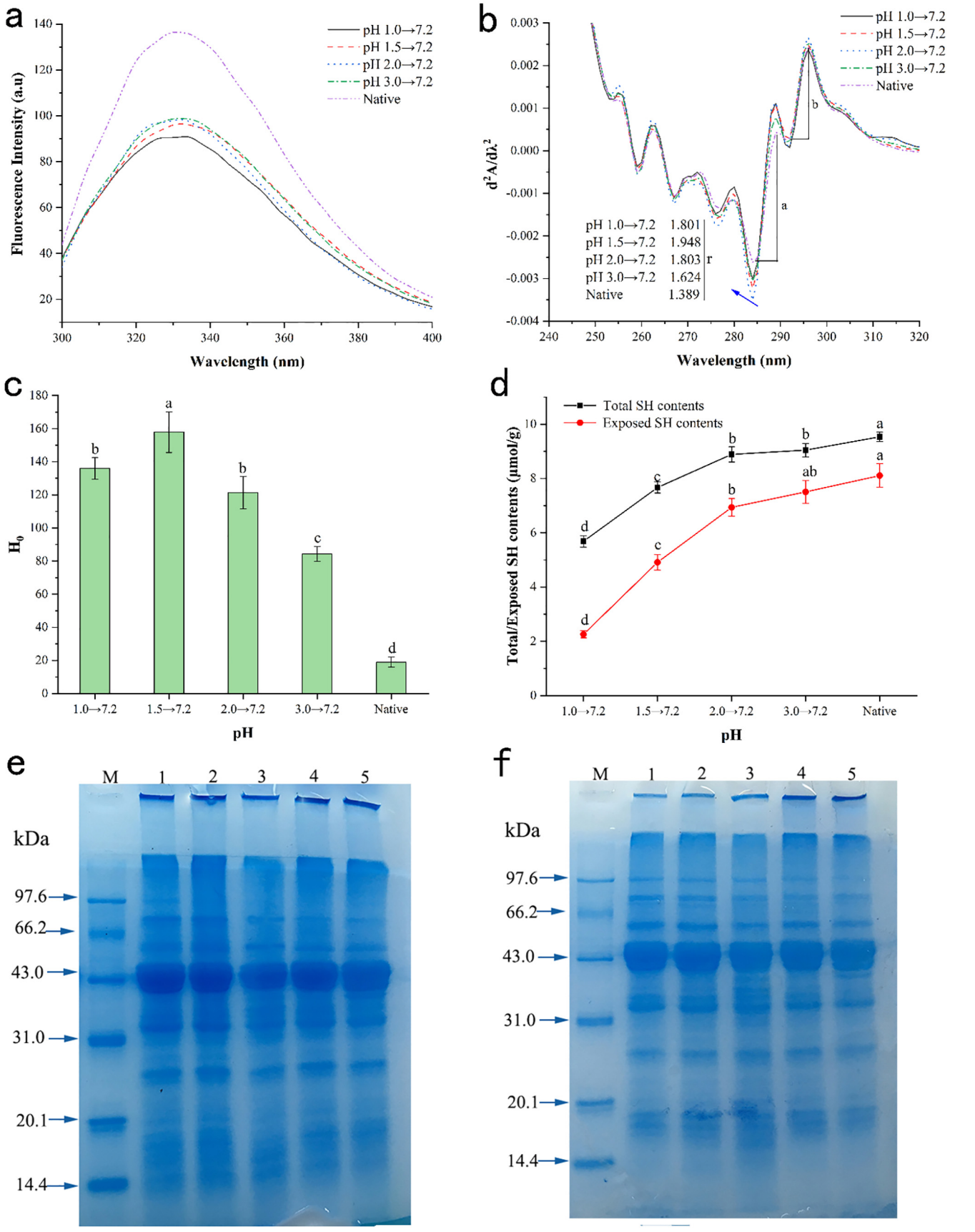

Fig. 2. Effects of low pH-shifting treatment on the (a) intrinsic fluorescence spectra, (b) Second-derivative UV spectra, (c) surface hydrophobicity (H_0), (d) total sulfhydryl (SH) and exposed SH contents, as well as SDS − PAGE profiles without (e) or with (f) β-mercaptoethanol of native and treated black turtle bean (*P. vulgaris* L.) protein isolates (Lane M: maker; Lanes 1–5: native, pH 3.0 → 7.2, pH 2.0 → 7.2, pH 1.5 → 7.2, and pH 1.0 → 7.2 shifting samples). Different letters (a–d) on the columns indicated the significant difference between each other at $P < 0.05$ level.

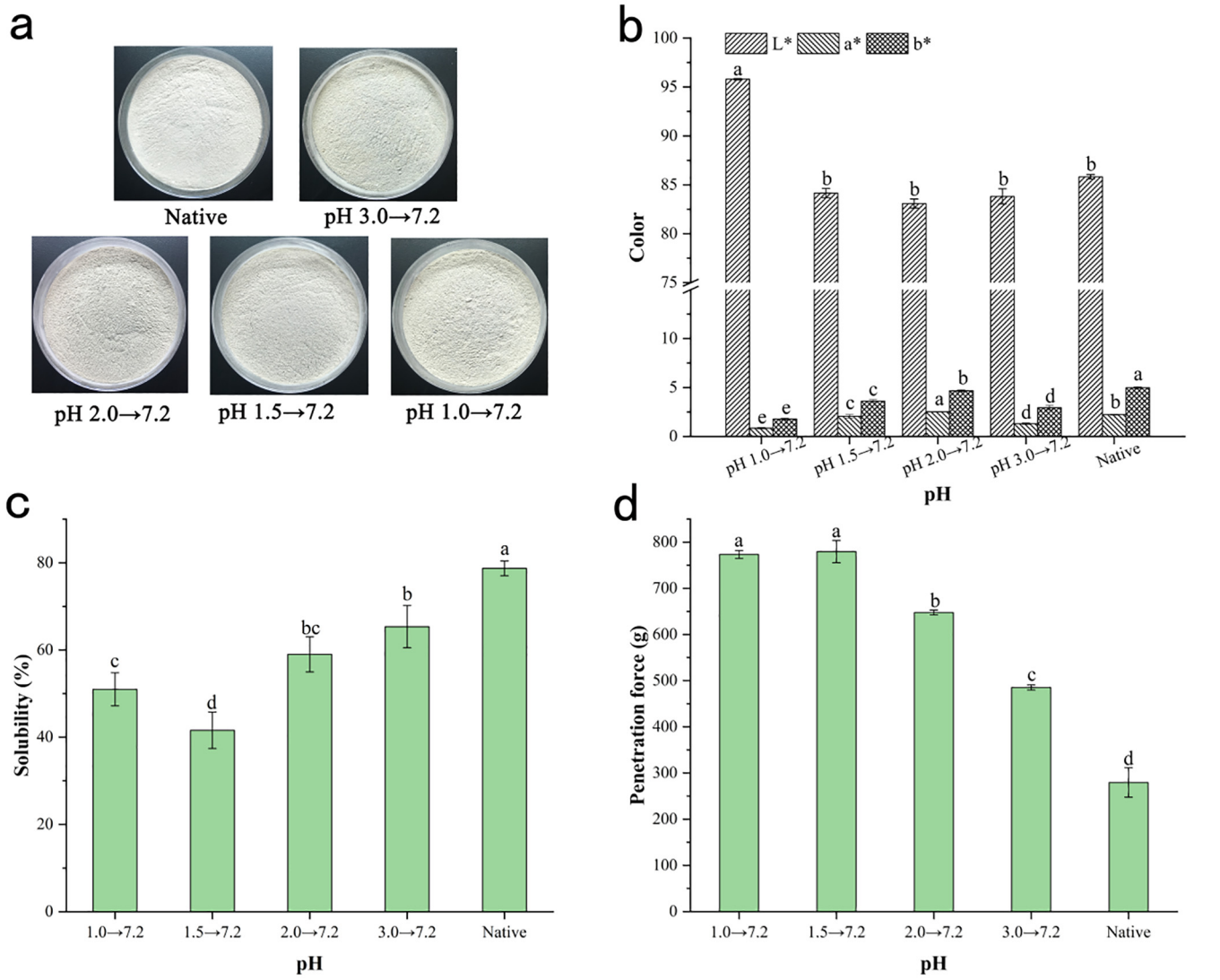

Fig. 3. The end product powder (a), colour (b), solubility (c) and gelling strength (d) of native and low pH-shifting black turtle bean (*P. vulgaris* L.) protein isolates. Different letters (a–e) on the columns indicated the significant difference between each other at $P < 0.05$ level.

3.1.5. Surface hydrophobicity

As presented in Fig. 2c, the surface hydrophobicity values (H_0) of all low pH-shifting proteins were significantly ($p < 0.05$) higher than that of native control, and in contrast to native protein, surface hydrophobicity was increased by 342.75%, 537.13%, 729.31% and 614.92% with the original pH decreasing from 3.0 to 1.0, respectively, indicated more hydrophobic residues on the molecular surface of protein after the low pH-shifting treatments (Liu et al., 2015; Wang et al., 2011). The increase in surface hydrophobicity was also observed in soy bean protein isolate (Jiang et al., 2009; Liu et al., 2015) and soy β-conglycinin and glycinin subjected to the low pH-shifting treatments (Jiang, Xiong, & Chen, 2011). Large numbers of the proteins in black turtle bean protein isolate might be deacetylated in low acidic pH environments, and low pH-shifting treatment might cause protein molecules to render an unfolding looser state with more hydrophobic residues exposed on the protein surface (Kristinsson & Hultin, 2004; Liu et al., 2015). It should be noted that the decrease of H_0 value for the pH 1.0 shifting sample might attribute to the protein denaturation and/or the molten-globule state conformational changes under the extreme acidic conditions (Jiang et al., 2011). Moreover, the results revealed that better emulsifying and foaming properties of the low pH-shifting protein sample would be predictable resulting from the exposure of hydrophobic binging sites (Jia, Wang, Shao, Liu, & Kong, 2017).

3.1.6. SH contents

The total SH content in native sample was 9.54 ± 0.18 μmol/g protein powder, and the value significantly decreased ($p < 0.05$) by 5.24, 6.81, 19.60, and 40.36% in the samples shifting pH from 3.0 → 7.2 to pH 1.0 → 7.2, while the exposed SH contents reduced to 92.60%, 85.57%, 60.54% and 27.87% of that of native control (8.11 ± 0.43 μmol/g), respectively (Fig. 2d). The reductions in the SH contents seemed to have association with protein unfolding, and more SH oxidation and SH/S−S interchange reactions occurred to form the disulfide bonds (Liu et al., 2015), and the further details were investigated by the gel electrophoresis analysis.

3.1.7. SDS-PAGE

According to previous researches (do Evangelho et al., 2017; Yin et al., 2010), black turtle bean proteins were mainly composed by four classes: (1) unnamed 11S protein, with molecular mass higher than 57 kDa; (2) Vicilin or Phaseolin (7–8S), with molecular mass of 47 kDa (α-type), 44 kDa, 25 kDa (β-type), and 21 kDa (γ-type); (3) lectin (31 kDa); and (4) α-amylase inhibitors (27, 18 and 17 kDa), depending on the subunit form. As shown in Fig. 2e, under the nonreducing conditions (without β-mercaptoethanol), extremely large proteins accumulated and the band intensity increased on the top of the stacking gel with the decreasing of original pH value, which might attribute to the

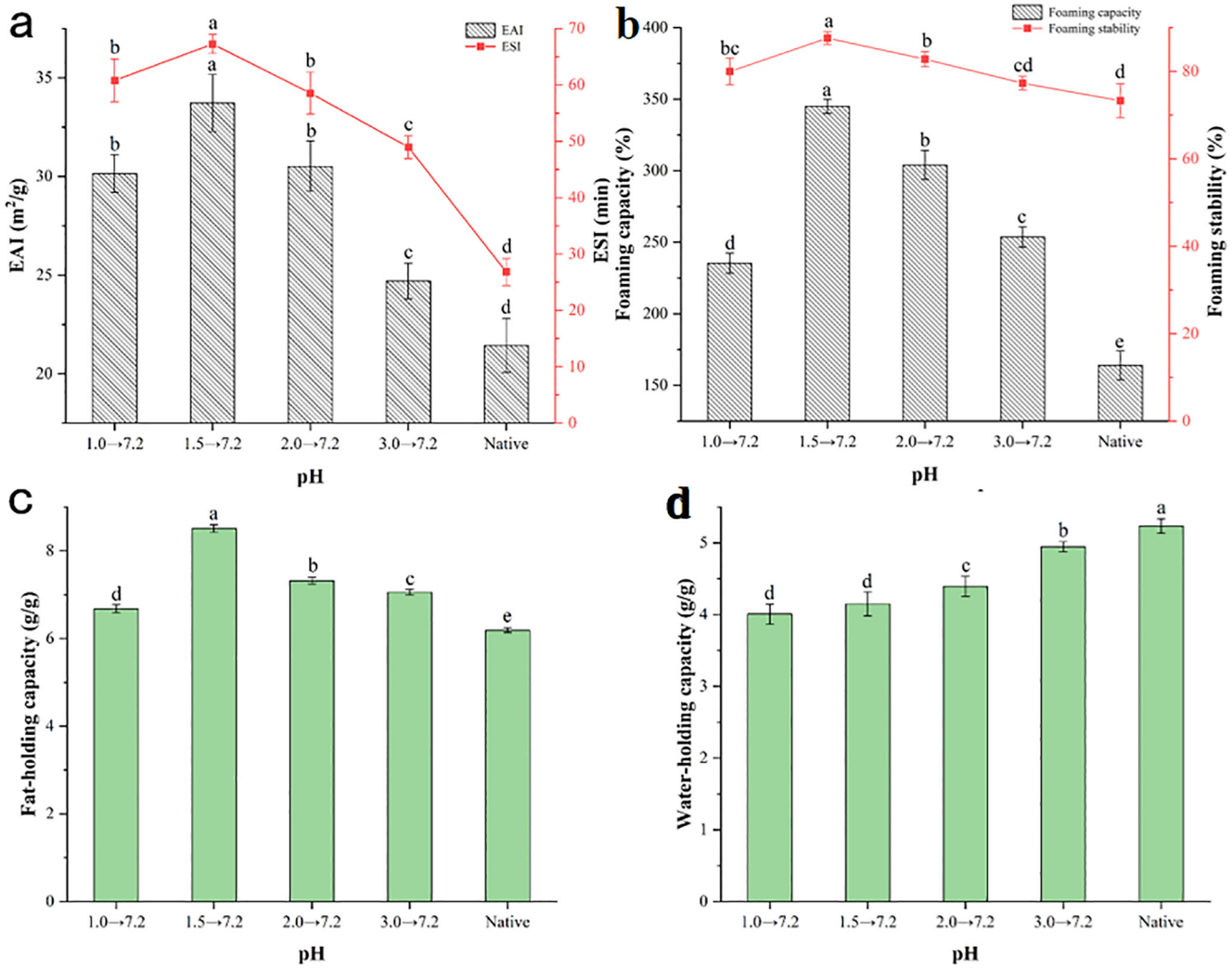

Fig. 4. The emulsifying properties (a), foaming properties (b), fat holding capacity (c) and water holding capacity (d) of native and low pH-shifting black turtle bean (*P. vulgaris* L.) protein isolates. Different letters (a–d) indicated significant difference between each other at $P < 0.05$ level.

disulfide cross-linking of protein molecules, confirmed the speculation in the SH content analysis. Furthermore, the band intensity of 11S protein with molecular mass higher than 57 kDa gradually decreased, and the protein bands around 97.6 kDa were not detectable after low pH-shifting treatments, indicating that 11S protein might be responsible for the formation of high MW protein polymers and aggregates (Jiang et al., 2009; Liu et al., 2015). Moreover, no obvious changes were obtained in the protein subunits below 57 kDa. When β-mercaptoethanol was employed in the SDS-PAGE analysis (Fig. 2f), the large MW polymers and aggregates were significantly dissociated, especially in native and pH 3.0 → 7.2 shifting samples. However, the large MW polymers and aggregates were still observed in the low pH-shifting samples, which might due to the increased surface hydrophobicity and S–S contents. Compared with the protein patterns of native control, the visible disappearance of the protein bands around 97.6 kDa in the pH-shifting 1.0 → 7.2 group under the reducing conditions also indicated the protein hydrolysis or protein composition alterations. Furthermore, the conformational variations might lead to favorable changes in the protein functional properties (Liu et al., 2015).

3.2. Functional property changes

3.2.1. Colour

No significant difference in external appearance was found among the freeze-dried protein powders by visual observations (Fig. 3a). As shown in Fig. 3b, negligible changes of L^* values have been found among the native low pH-shifting samples, while pH 1.0 → 7.2 shifting sample exhibited an evident increase in brightness, which might be attributed to the destruction of darker coloured compounds in lower pH environments (Wang & Zhong, 2014). On the other hand, both the a^* and b^* values of all the samples were found at a lower level ($a^* < 3$, $b^* < 5$) without remarkable colour changes.

3.2.2. Solubility

As shown in Fig. 3c, the solubility of native control was 78.74%, while the solubility significant dropped ($P < 0.05$) by 16.99, 25.07, 47.18 and 35.24% for the samples treated with pH 3.0 → 7.2 shifting to pH 1.0 → 7.2 shifting, respectively. The decrease in solubility might due to the protein molecules unfolding and more exposure of buried hydrophobic groups, which would decrease the intermolecular interactions. Similar solubility reduction also was also found by Jiang et al. (2009) and Liu et al. (2015), respectively, when soy protein isolate was subjected to low acid pH-shifting treatment. Since the proteins after pH 1.0 shifting treatment would be more flexible, the improvement of solubility might be resulted from the balance of the hydrophobicity and hydrophilcity as well as the hydratability of the proteins to the water (Jiang & Ding, 2017).

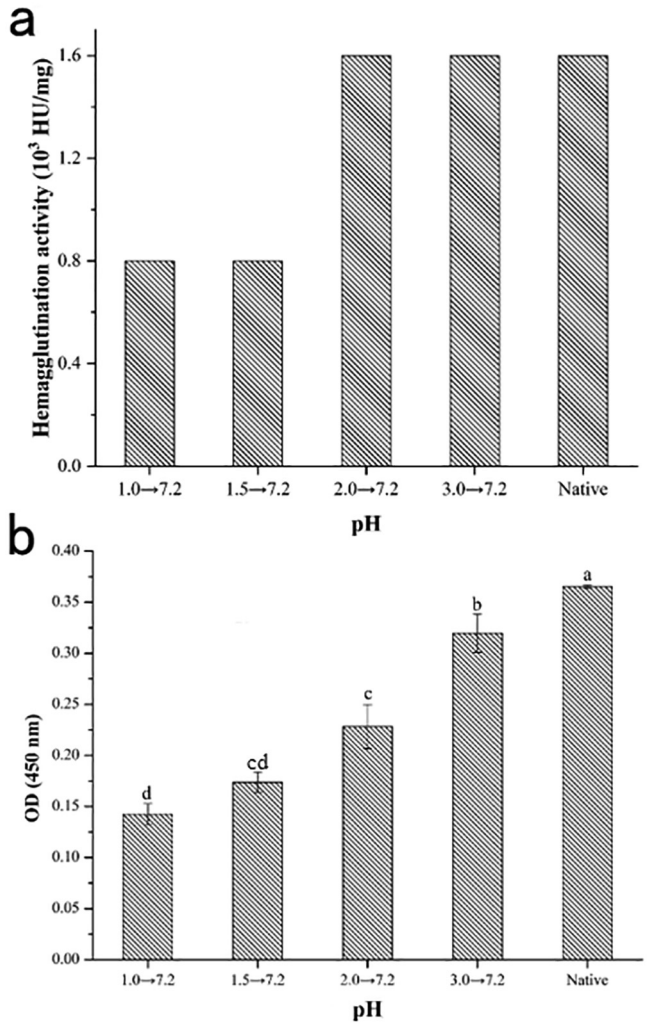

Fig. 5. The hemagglutination activity (a) and immunoreactivity (b) of native and low pH-shifting black turtle bean (*P. vulgaris* L.) protein isolates. Different letters (a–d) on the columns indicated the significant difference between each other at $p < 0.05$ level.

3.2.3. Gelling properties

Compared with the native control, the gel strength of black turtle bean protein isolate sample increased by 73.57, 131.66, 178.86 and 176.61%, respectively, after the pH-shifting from 3.0, 2.0, 1.5 and 1.0 to 7.2, and the maximal penetration forces of 773.29 ± 8.40 g and 779.57 ± 24.09 g were obtained in the pH 1.0 and 1.5 shifting samples (Fig. 3d). It has been proven that the forming of three-dimensional gel network in the thermal gelation processes was resulting from the rearrangements and aggregation of hydrophobic amino acid groups (Krešić, Lelas, Jambrak, Herceg, & Brnčić, 2008). Thus, the protein unfolding and exposure of hydrophobic amino acid groups induced by the low pH-shifting treatment would be beneficial to the gelation formation (Liu et al., 2015), and an ideal balance of hydrophilic and hydrophobic amino acid residues might be obtained in the pH 1.0 → 7.2 and 1.5 → 7.2 shifting protein isolates. Furthermore, since no visible differences could be observed between the pH 1.0 → 7.2 and pH 1.5 → 7.2 treated groups, the pH 1.5 shifting treatment seemed to be more suitable in facilitating protein gelling formation with the higher gel strength, and the results were similar with the previous reports (Jia et al., 2017).

3.2.4. Emulsifying activity and emulsion stability

The EAI and ESI changes presented similar increasing tendencies with the surface hydrophobicity analysis above (Fig. 4a), and both the EAI and ESI values of all treated samples were significantly improved ($P < 0.05$). With respect to native control, the largest increase percentages of EAI (57.32%) and ESI (60.16%) were both found in the pH 1.5 → 7.2 shifting samples. According to the previous studies, the buried hydrophobic residues would be exposed due to the decrease of α-helix content and the increase of protein flexibility (Wang et al., 2011), and an interfacial membrane might be formed to disperse the oil droplets because of the increasing of the protein hydrophobicity (Zhang et al., 2014). Similar emulsifying properties were also obtained in the soy protein isolate treatments (Jiang et al., 2009; Jiang et al., 2011), and the pH 1.5 shifting treatment might result in the maximum exposure of hydrophobic sites with an ideal balance of hydrophilic and hydrophobic amino acid residues (Farrell et al., 2002).

3.2.5. Foaming capacity and foaming stability

According to Fig. 4b, significant increases ($P < 0.05$) in FC and FS were observed in low pH-shifting protein samples. Similar with the EAI and ESI analyses, the protein unfolding and exposure of hydrophobic groups induced by the low acidic conditions would be essential for adsorption of protein onto the water and air interfacial molecules (Režek Jambrak, Lelas, Mason, Krešić, & Badanjak, 2009), and the buried hydrophobic groups might be responsible for the development of FC and FS. The protein isolates recovered from the pH 1.0 treatment might be suffered from more stronger intramolecular electrostatic repulsions leading to the dissociation of protein complexes and unfolding of the protein, thereby the decreases in the hydrophobic functions would be closely related to the conformational changes and/or protein hydrolysis (Jiang & Ding, 2017).

3.2.6. Fat holding capacity

As expected, significant increase ($p < 0.05$) in FHC was observed in all samples subjected to low pH-shifting treatments, and the highest FHC value (8.51 ± 0.09 g/g) was obtained in the pH 1.5 → 7.2 shifting protein sample (Fig. 4c). The increases of FHC could be largely attributed to numerous exposure of buried hydrophobic amino acid residues, resulting in more interactions with oil droplets (Yongsawatdigul & Hemung, 2010). Evidently, different acidic incubation pH values would lead to various unfolding states of the protein structures that might modify functionalities of the protein (Kristinsson & Hultin, 2003a, 2003b), and the highest FHC value of the sample subjected to pH 1.5 → 7.2 shifting treatment could be explained by the maximum exposure of the hydrophobic groups.

3.2.7. Water holding capacity

As shown in Fig. 4d, the WHC of native sample was 5.23 ± 0.10 g/g protein, and it decreased significantly ($P < 0.05$) in the low pH-shifting treated samples. In a detail, the WHC values of pH 3.0 → 7.2, 2.0 → 7.2, 1.5 → 7.2 and 1.0 → 7.2 shifting samples reduced to 94.64, 83.94, 79.35 and 76.67% of that of native control, respectively. WHC of protein molecules could be affected by some intrinsic factors including amino acid composition, protein conformation and surface polarity/hydrophobicity. Obviously, the exposure of hydrophobic groups would lead to the decrease in the interaction with water, resulting in dehydration of protein molecules, meanwhile, the formation of high MW protein polymers and aggregates observed in the SDS-PAGE analysis would result in the decreasing concentration of soluble proteins, which would also lead to poor interactions with water molecules (Deng et al., 2011; Yuliana, Chi, Huynh, Ho, & Ju, 2014). It was obvious that not only the hydrophobic residues were exposed, but also the hydrophilic groups were also enhanced in the pH 1.0 shifting protein isolates (Fig. 3c), which would be the major reason for the little reduction in the WHC of the acidic pH treated sample.

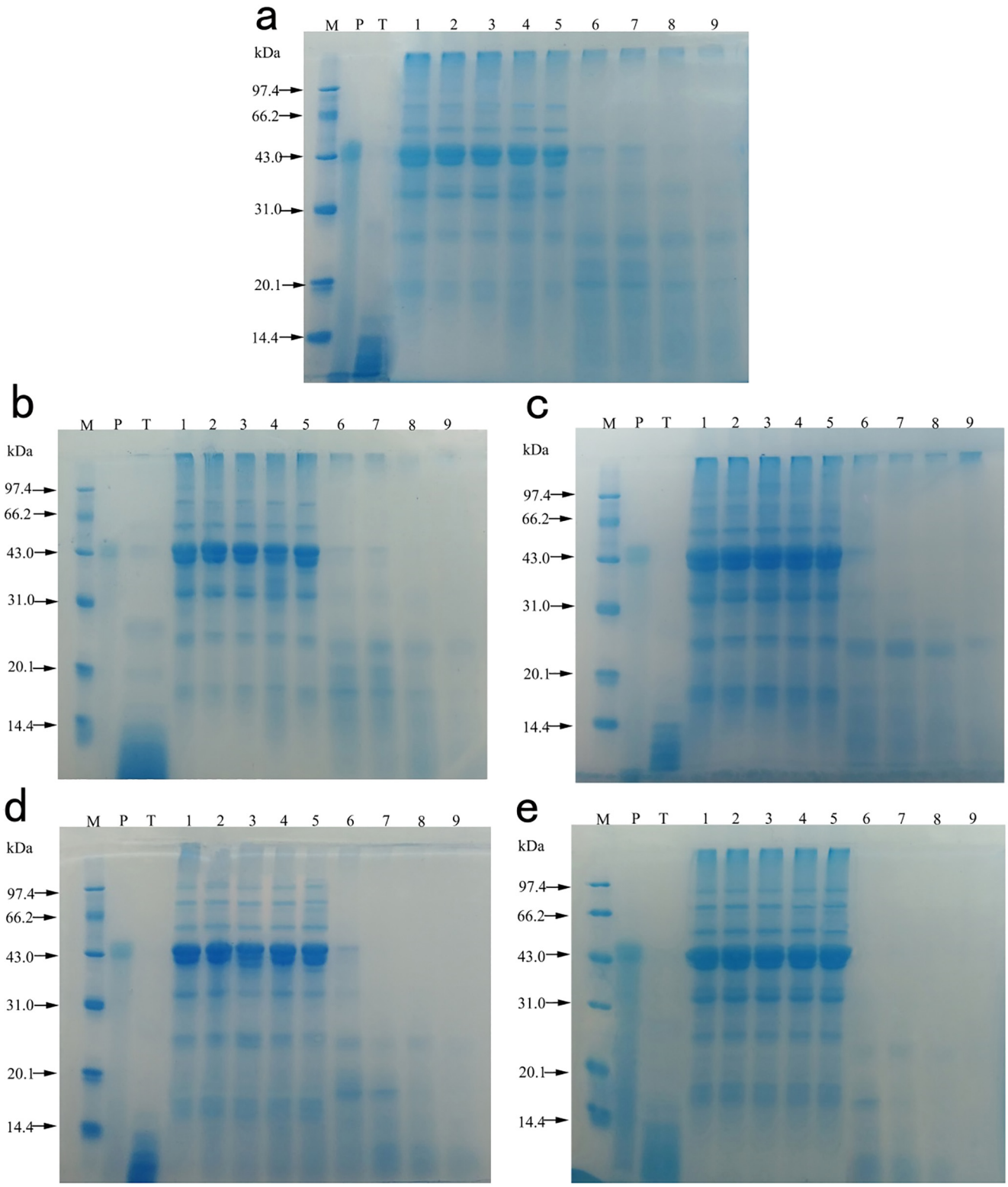

Fig. 6. The digestion stabilities of native (a) and low pH-shifing (b, pH 3.0 → 7.2; c, pH 2.0 → 7.2; d, pH 1.5 → 7.2 and e, pH 1.0 → 7.2) black turtle bean (*P. vulgaris* L.) protein isolates in SGF and SIF digestion process, respectively. Lane M, protein marker; Lane P, pepsin; Lane T, trypsin; Lanes 1–5, simulated gastric digestion for 0, 10, 20, 30 and 60 min, respectively; Lanes 6–9, simulated intestinal digestion for 5, 10, 30 and 60 min, respectively.

3.3. Hemagglutination activity

The reaction of the lectin with carbohydrates or glycoproteins on the surface of the erythrocytes could be determined by the hemagglutination activity analysis (He et al., 2018). As shown in Fig. 5a, the hemagglutination activity of treated black turtle bean protein isolate remained stable decreased to pH 2.0 shifting samples, suggesting the structural perturbation would be far away the regions of specific sugar-binding sites in the pH adjustments. While 50% loss of the hemagglutination activity in relation to native sample was found in pH 1.5 → 7.2

and pH 1.0 → 7.2 shifting protein sample, respectively, indicated that some sugar-binding sites might be disrupted during the low pH recovery because of the conformational changes (Zhao et al., 2019). Since the hemagglutination activity from lectin was closely related with allergencity of legumes (Nasi, Picariello, & Ferranti, 2009), the decreasing results might indicate the safety of black turtle beans protein isolate subjected to the low pH-shifting treatments.

3.4. Immunoreactivity

The immunoreactivity of the black turtle bean protein isolate was evaluated by ELISA assays (Fig. 5b). Generally, the IgE binding capacity decreased significantly ($P < 0.05$) with the reduction of incubation pH values from 7.2 to 1.0, specifically, the immunoreactivity reduced by 11.11, 36.11, 52.78 and 73.33% of native control in the samples treated with pH 3.0 → 7.2 to pH 1.0 → 7.2 shifting, respectively, indicated that the low pH-shifting treatments would promote the antigenicity reduction of the protein samples. Based on the structural analyses above, the protein secondary structure alteration and unfolding in low acidic conditions might disrupt the surface epitopes, resulting in the loss in IgE recognition ability (Zhao et al., 2019). Similarly, sharp reduction in antigenicity was also found in low pH-shifting tropomyosin from short-neck clam (*Ruditapes philippinarum*) (Lin et al., 2015).

3.5. In vitro digestion stability

High immunoreactivity was well related with the anti-digestibility of protein, which could provide the potential chance to induce an immune response on a cellular level (Apostolovic et al., 2016). SDS-PAGE was applied to evaluate the *in vitro* digestibility, and the results were displayed in Fig. 6. In SGF digestion process, there were no obvious changes in band intensity of native and low pH-shifting samples with the expanding of digestion time, suggesting a consistent resistance to pepsin digestion. Similar results also were observed in our previous investigation of the *in vitro* digestibility of native and PEGylated black turtle bean protein isolate (He et al., 2018). However, in the continuous SIF digestion process, significant decrease was observed in the intensity of native protein band, with the generations of new digested bands around 28 and 18 kDa, and the band would be undetectable at the end of 60 min digestive process (Fig. 6a), indicated that the protein isolate could be digested in small intestine. It was interesting that the new generated bands around 28 and 18 kDa were digested after a 30 min incubation in the pH 3.0 → 7.2 and 2.0 → 7.2 shifting samples (Fig. 6b and c), the bands would be almost invisible after only in 10 and 5 min digestion for the protein samples subjected to pH 1.5 → 7.2 and 1.0 → 7.2 shifting treatments, respectively (Fig. 6d and e). The less retention time of the protein bands during *in vitro* simulated digestion process confirmed the improvement of digestibility for the low pH-shifting black turtle bean protein isolates. Taken together the structural analyses, the protein might be in a looser state with unfolded conformation after the low pH-shifting treatments, then more trypsin cleavage sites would be exposed, which might make the protein be more susceptible to proteolytic attack (He, Simpson, Ngadi, & Ma, 2015; Zhao et al., 2019), and the improvement of digestive characteristic would be beneficial to the immunoreactivity reduction during the legume protein consumption.

4. Conclusion

In conclusion, our present investigation indicated that structural rearrangement with the exposure of buried hydrophobic amino acid residues would be occurred in the black turtle bean (*P. vulgaris* L.) protein isolate subject to the low pH-shifting treatments, which might be contributed to the enhancements of emulsifying properties (EAI and ESI), gelling properties, foaming properties (FC and FS) and FHC, while would cause the reduction of solubility and WHC. Meanwhile, the structural modification might destroy sugar-binding sites, surface epitopes and expose more protease cleavage sites, which would lead to the evident decreases of hemagglutination activity and potential immunoreactivity, meanwhile, the *in vitro* digestibility was improved. Accordingly, the low pH-shifting treatment might have great potential to produce functionality-improved black turtle bean (*P. vulgaris* L.) protein isolate with a high level of consumption safety, and the pH-shifting treatment would expand the utilities of *P. vulgaris* L. protein isolates.

CRediT authorship contribution statement

Shudong He: Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition. **Jinlong Zhao:** Investigation, Methodology, Writing - original draft. **Xiaodong Cao:** Validation, Resources, Funding acquisition. **Yongkang Ye:** Validation, Resources, Funding acquisition. **Zeyu Wu:** Validation, Resources. **Junyang Yue:** Validation, Resources. **Liu Yang:** Methodology. **Risheng Jin:** Methodology, Funding acquisition. **Hanju Sun:** Data curation, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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