



Improving emulsification properties of alkaline protein extract from green tea residue by enzymatic methods

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ARTICLE INFO

Handling Editor: Xing Chen

Keywords:

Leafy biomass
Protein degradation
Viscozyme® L
Pepsin
Alkaline protease
Emulsification capacity

ABSTRACT

Alkaline extraction is an important process in the integrated biorefining of leafy biomass to obtain protein, but the resulting alkaline protein extract (APE¹) may have poor emulsification properties for food applications. In this study, the components in the APE fractionations obtained by size exclusion chromatography were determined. The emulsification properties of APE were determined using oil/water with a ratio of 7:3. Whey protein and soybean protein isolate were used as controls while enzymes were used to improve APE's emulsification properties. The results showed that the APE could be divided into three fractions with protein content of 83, 56, and 34%. Carbohydrates mainly derived from homogalacturonan pectin were mostly in Fraction 2, while Fraction 3 consisted of peptides, oligosaccharides, and free polyphenols. The APE had similar emulsification capacity and emulsification stability as those of whey protein and soybean isolate. The emulsion made by the APE had a creaming index of 92% with emulsification activity index value of $44 \text{ m}^2 \text{ g}^{-1}$, and these numbers could retain after storing at 25 °C for 15 days. The emulsification properties of the APE can be further improved by carbohydrate degradation. With the use of Viscozyme® L, the emulsification activity index value of treated APE was increased by 60%, and then still retained at $67 \text{ m}^2 \text{ g}^{-1}$ after storing for 15 days. Treated by either pepsin or alkaline protease, the emulsification properties of APE were decreased, suggesting the key role of protein in APE for emulsification.

1. Introduction

The exploration of new protein sources for human consumption could help satisfy the demands of the growing population while reducing our ecological footprint (Zhang et al., 2014). Protein from tea leaf residues is a good source of protein that accounts for 20–30% of tea residue in dry weight. It has been shown to have a similar amino acid profile to soybean protein (Zhang et al., 2012) as well as potential use as an emulsifier (Di Stefano et al., 2018; Wu et al., 2013). Preliminary test suggested that tea leaf protein can be obtained effectively using a biorefinery concept (Zhang et al., 2016a, 2016b), and the remained challenge is to improve protein functionalizes, including emulsification properties (Gao et al., 2020) to increase the its marketing value.

In the biorefinery, alkali was used for protein extraction (Zhang et al., 2016a, 2016b). However, although more than 95% of protein can be extracted, protein functionalities, such as emulsification properties, can be reduced or even totally lost (Hou et al., 2017). The reduction of emulsification property can be attributed to protein denaturation (Liu et al., 2011) and degradation (Zhang et al., 2018), or the reaction of the proteins with other non-protein components, including polyphenols (Feng et al., 2017) and carbohydrates. The amino group of proteins can react with the phenolic hydroxyl group of polyphenols to generate protein-polyphenol complexes, thus reducing the physical and chemical properties of proteins (Rohn, 2014). The carbohydrate-protein complex can either originate naturally from the leaves or from the reaction of reducing sugars and amino acids (Maillard reaction) during alkaline

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¹ alkaline protein extract: APE.

<https://doi.org/10.1016/j.crf.2022.07.016>

Received 3 May 2022; Received in revised form 9 July 2022; Accepted 31 July 2022

Available online 8 August 2022

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extraction (Yang et al., 2010). The influence of carbohydrates on proteins is complicated, with both negative and positive effects reported (Hirsch et al., 2019; Gentile, 2020).

To explore the potential use of alkaline-extracted leaf protein as an emulsifier, the composition of the main components and their relationships, particularly the possible reactions between proteins and polyphenols or carbohydrates, should be analyzed. Separation techniques are typically used in alkaline extraction (Valdés et al., 2020); however, this approach would be challenging for the extraction of leaf proteins due to the complexity of alkaline protein extracts (Zhang et al., 2015). In contrast, enzymatic methods might prove more effective. Enzymes can be used to specifically hydrolyze proteins or carbohydrates, followed by the analysis of their hydrolysate compositions and their influence on emulsification property. By associating the degradation of target components with the variation in the composition and emulsification properties of alkaline protein extracts, the key components for emulsification can be found without separation. In addition, the conditions of enzyme hydrolysis can be controlled to further improve protein emulsification properties. Proteins can be partially degraded, thereby improving the emulsification properties of protein products due to the exposure of more hydrophilic or hydrophobic groups (Jung et al., 2010; Farooq et al., 2022).

Thus, the aim of this study was to characterize the alkaline protein extract (APE) from green tea residue (GTR) and improve its emulsification properties using enzymatic methods that specifically degrade carbohydrates or proteins in different APE fractions. The compositions of the APE, including protein, carbohydrate, and polyphenol contents, were determined. The APE was then fractionated by size-exclusion chromatography based on its molecular weight distribution, and the main components in each fraction were identified and quantified. Furthermore, Viscozyme® L was used to hydrolyze carbohydrates, pepsin was used to degrade peptide bonds after aromatic or acidic amino acids, and alkaline protease was used to degrade peptide bonds after hydrophobic acids. Finally, the emulsification properties of the APEs (with and without treatments) were determined. Based on the results, the origination of proteins in each fraction, the possible reaction of proteins with polyphenols or carbohydrates, and their influence on protein emulsification were discussed.

2. Materials and methods

2.1. Materials

The GTR was gifted by Fujian Damin Food (Zhangzhou, China) and was produced by extracting tea leaves (*Camellia sinensis*) in water at 85 °C for 45 min. The GTR was then dried at 60 °C for 12 h. To obtain a protein extract, the GTR (20 g) was extracted in 400 mL 0.2 M NaOH at 95 °C for 3 h, after which the supernatant (protein extract) was collected by centrifugation. After acid purification at pH 3.5 using 0.1M HCl (Zhang et al., 2015), the precipitate was collected by centrifugation, and then thoroughly dissolved in water by adjusting pH to 7 using 0.1M NaOH. After a repeat of acid precipitation, the extract was freeze-dried to obtain APE, which can be store for a long time for further analysis.

Whey protein (80% purity), soybean protein isolate (90% purity), alkaline protease (800 U mg⁻¹, from *Bacillus licheniformis*), pepsin (800 U mg⁻¹), D-xylose, D-galactose, D-mannose, D-glucose, D-arabinose, and tea polyphenols were purchased from Yuanye Reagent, Shanghai, China. Viscozyme® L (1000 U mg⁻¹) was purchased from Sigma-Aldrich (Shanghai branch, China). Other chemicals (analytical grade) were purchased from Sinopharm Chemical Reagent (Shanghai, China).

2.2. Sample treatments

2.2.1. Fractionation of APE

The molecular weight distribution of the samples was analyzed by an SEC300 column (Thermo Fisher, USA). After filtration through a 0.22

µm membrane, 100 µL of sample was injected into a high-performance liquid chromatograph (HPLC) (Agilent 1100) and detected by a DAD detector (280 nm) at 25 °C with a flow rate of 0.25 mL min⁻¹.

Fractions were collected according to the four peaks separated by size exclusion chromatography. Fraction 1 was the eluent between 10 min and 13 min, Fraction 2 was the eluent between 13 min and 20 min, and Fraction 3 was the eluent between 20 min and 23 min. To obtain enough samples, the loading APE was with 10 mg mL⁻¹, and 10 rounds of separation and collection were applied. The collected solutions were merged and freeze-dried and stored for future use.

2.2.2. Extraction of free polyphenols

Ten milliliters of 50% ethanol solution was added to 1 g of APE, and the mixture was then incubated in a Thermomixer at 60 °C for 1 h with a shaking speed of 1000 rpm min⁻¹. The supernatant was then collected by centrifugation, and the total amount of free polyphenols was ready for polyphenol determination. The solid part was washed twice in 50% ethanol, followed by a freeze-drying step. The dried solid sample was then ready for further analysis.

2.2.3. Dialysis

Ten milliliters of solution (50% ethanol for polyphenol, demi-water for monosaccharides) was added to 1 g of APE, following which the mixture was transferred to a dialysis bag with a 3 kDa molecular weight cut off (MWCO). The dialysis was conducted in a closed and oxygen-free environment under ambient conditions for 24 h using 40 mL 50% ethanol or 40 mL demi-water as external solutions. After dialysis, the dialysate of 50% ethanol was collected for polyphenol determination, while the retention was used for monosaccharide determination. Notably, samples containing Viscozyme® L were preliminarily inactivated at 90 °C for 5 min.

2.2.4. Enzymatic methods

A preliminary experiment was performed to ensure that enzyme hydrolysis was complete (see Supplementary Data Fig. S1). The protocols to hydrolyze 10 mg mL⁻¹ APE were as follows.

- 1) 0.1 M HCl was used to adjust the pH to 4, following which 1 mL APE solution was mixed with Viscozyme® L (10 U) and incubated in a thermomixer at 35 °C for 2 h.
- 2) 0.1 M HCl was used to adjust the pH to 3.5, following which 1 mL APE solution was mixed with pepsin (20 U) and incubated in a thermomixer at 35 °C for 2 h.
- 3) 0.1 M NaOH was used to adjust the pH to 8.5, following which 1 mL APE solution was mixed with alkaline protease (20 U) and incubated in a thermomixer at 35 °C for 2 h.

After hydrolysis, the pH of all samples was adjusted to 7 using 0.1 M NaOH or 0.1 M HCl, followed by dilution using a demi-water to an APE concentration of 1 mg mL⁻¹. The hydrolysates were then ready for further analysis.

2.3. Determinations

2.3.1. Protein content

The protein content (g L⁻¹) was determined by a total organic carbon analyzer (TOC-L, Shimadzu, Japan). The TOC analyzer automatically measures all nitrogen concentrations in the sample, including non-protein nitrogenous substances such as caffeine, chlorophyll, and theobromine. According to a previous study (Zhang et al., 2015), a conversion factor of 5.4 was used to calculate the protein concentration.

2.3.2. Total carbohydrate content

Total sugar content was determined by the anthrone-sulfuric method at 625 nm (Yemm and Willis, 1954). Glucose was used as a reference in a concentration range from 0 to 50 mg L⁻¹.

2.3.3. Ash content

Samples were pre-weighed in a weighted crucible. After preheating in the oven at 120 °C for 2 h, the crucible was transferred to a 550 °C furnace for 16 h to burn off all the organic matter. The crucibles were weighed after cooling down in a desiccator. Ash contents were calculated as weight percentages of the starting material.

2.3.4. Polyphenol content

The content of polyphenols was determined according to the ferrous tartrate method (Hosseinzadeh et al., 2013), whereby samples were reacted with ferrous tartrate to form a purple-blue complex, and the absorbance was measured at 540 nm. Tea polyphenols (purity 98%, Yuanye Biological Co., Ltd., Shanghai) were used as a standard sample with a concentration range of 0–0.5 mg mL⁻¹.

To quantify and identify bound polyphenols, 1 g solid sample prepared as in 2.2.2 was mixed with 10 mL 2 M HCl. Referring to Aludatt's method (Alu'Datt et al., 2013), the mixture was hydrolyzed at 25 °C for 8 h. After hydrolysis, the pH of the hydrolysate was adjusted to higher than 3. The supernatant was collected and dialyzed using 50% ethanol, as described in 2.2.3.

2.3.5. Identification of polyphenols

The polyphenols were identified by liquid chromatography-mass spectrometry (LC-MS) (TSQ Quantum Access MAX, Thermo Fisher Scientific, USA) using C18 as a separation column (5 μm 120 Å 4.6 × 250 mm, Thermo Scientific, U.S.) (Alu'Datt et al., 2013). Using 100% methanol and 0.2% as mobile phases, the separation protocol was as follows: 0–50 min (from 5% methanol and 95% acetic acid to 80% methanol and 20% acetic acid) and 50–60 min (from 80% methanol and 20% acetic acid to 5% methanol and 95% acetic acid), with a flow rate of 0.75 mL min⁻¹. The sample injection volume was 20 μL and the column temperature was 25 °C, and the fractions were determined at a wavelength of 280 nm. Based on the results, polyphenols were identified using The Human Metabolome Database (HMDB) (<https://hmdb.ca/>).

2.3.6. Galacturonic acid content

According to the carbazole colorimetric method (Kumar and Kumar, 2017), 1.5 mL concentrated sulfuric acid (98%) was added to 100 μL of sample in 2-mL Eppendorf centrifugation tubes, which were cooled in an ice water bath. After the addition of 50 μL ethanol solution with 0.1% carbazole, the final mixture was mixed and incubated in a Thermo-Mixer® C (Eppendorf, Germany) at 60 °C for 1 h. After the reaction, the sample was cooled using an ice water bath and was ready for analysis at 530 nm. D-(+)-Galacturonic acid (purity 97–100%, Sinopharm Chemical Reagent, Shanghai, China) was used as a standard with a concentration range of 0–200 μg L⁻¹.

2.3.7. Neutral monosaccharide composition

To determine the composition of monosaccharides, samples were hydrolyzed by 2 M trifluoroacetic acid solution at 120 °C for 5 h. After hydrolysis, the samples were collected. The neutral monosaccharide composition was determined by gas chromatography (6890 A, Agilent Technologies, Santa Clara, CA, USA) (Zhang et al., 2021).

2.3.8. Emulsification capacity

The oil/water ratios of 3:7, 5:5, 7:3 and 9:1 for emulsification were pretested as shown in Supplementary Data Fig. S2, by which 7:3 was selected. Protein solution (1.2 mL), including APE, whey protein, and soybean protein isolate, was adjusted to pH 7 using 0.1 M NaOH or HCl, and then mixed with 2.8 mL oil with a final protein concentration of 1 mg mL⁻¹.

The mixture was then emulsified by a homogenizer (T10 basic, IKA, Germany) with a speed of 20000 r min⁻¹ for 2 min. The creaming index (CI) was calculated as described in the below equation (Xu et al., 2021):

$$CI (\%) = \frac{H_s}{H_t} \times 100 \quad (1)$$

where H_t and H_s are the total volume of solution and emulsion layer volume, respectively.

The emulsifying activity index (EAI) was determined by a spectrophotometer (725N, INESA Analytical Instrument Co., Ltd, Shanghai, China) (Aziz et al., 2020). Emulsion (20 μL) was thoroughly mixed with 2 mL 1% SDS solution, and the absorbance of mixture was determined by spectrophotometer at 500 nm (A500). The value of EAI was calculated by Equation (2).

$$EAI (m^2 \cdot g^{-1}) = \frac{2 \times 2.303 \times A_{500} \times N \times 10^{-4}}{\theta C} \quad (2)$$

N: dilution times (101); θ: percentage of oil; C: protein concentration (g mL⁻¹).

2.4. Statistics

All extractions were triplicated and each sample was measured twice (n = 6), and the standard deviations were calculated. Data were analyzed by one-way ANOVA, and statistical analysis was carried out using Microsoft excel.

3. Results and discussion

3.1. Characterization of the APE

3.1.1. APE fractions and analysis of their compositions

The composition of the APE, including protein, total carbohydrates, polyphenols, and ash, was determined, and the results are presented in Fig. 1a. In the APE, protein was the major component that accounted for 52% of the total dry matter, with the contents of carbohydrates, ash, and polyphenols accounting for 18%, 11%, and 3%, respectively. The undetermined components were mainly quinones (oxidation of polyphenols), lipids (from wax), organic acids, and lignin fragments (Hernández et al., 2006; Shirai, 2019; Zhang et al., 2015). These components constituted about 18% of the APE. The molecular weight distribution of the APE was determined by size exclusion chromatography (SEC), and the results are shown in Fig. 1b. Three major fractions were

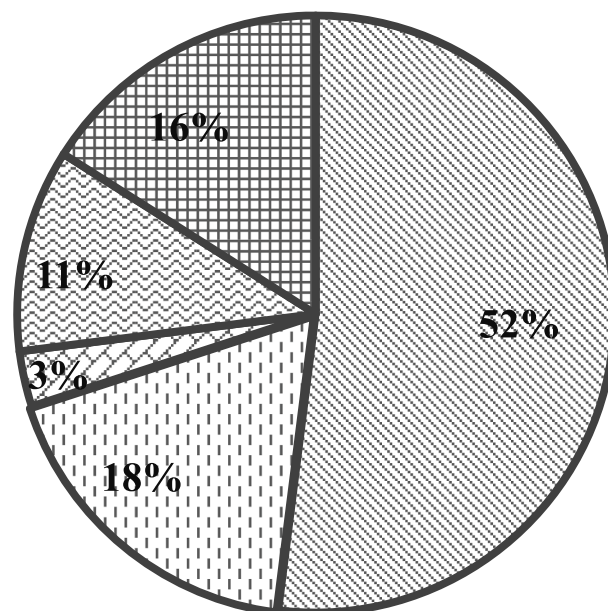


Fig. 1a. Composition of the alkaline protein extract. fx1: Protein; fx2: Carbohydrate; fx3: Polyphenol; fx4: Ash; fx5: Undetermined components.

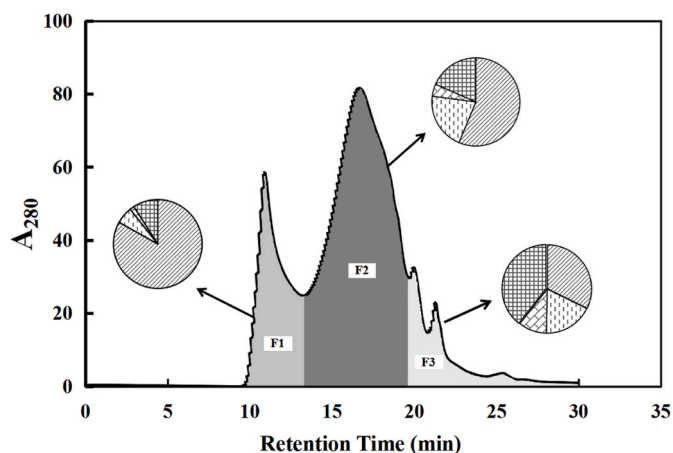


Fig. 1b. Size exclusion chromatogram analysis (0.05M, pH 7 PBS buffer, flow rate 0.5 mL min^{-1} , 30 min, $25 \text{ }^\circ\text{C}$) of the alkaline protein extract and the compositions of three fractions. fx6: Protein; fx7: Carbohydrate; fx8: Polyphenol; fx9: Undetermined components.

detected in the APE, and the molecular weight distributions of these fractions were Fraction 1 $> 100 \text{ kDa}$, $100 \text{ kDa} >$ Fraction 2 $> 3 \text{ kDa}$, and $3 \text{ kDa} >$ Fraction 3, which were collected as indicated in Fig. 1b.

The dry matter of the three fractions was about 24% for Fraction 1, 60% for Fraction 2, and 16% for Fraction 3, and the compositions of these fractions were determined and presented in Fig. 1b. Fraction 1 had the highest protein content of 83%, followed by Fraction 2 with 56% protein, while Fraction 3 had the lowest protein content of only 32%. The amount of protein in Fraction 1 was about one third of the total protein in the APE, with molecular weights higher than 100 kDa . It can be speculated that the Fraction 1 protein was mainly ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Nathan et al., 2013). RuBisCO is a well-known leaf protein with a molecular weight of about 560 kDa , accounting for 20–50% of total leaf protein (Hou et al., 2017; Spreitzer and Salvucci, 2002). In Fraction 2, the carbohydrate and polyphenol contents reached up to 21% and 6% of the dry matter, respectively. Many plant proteins are within this range, such as glycoproteins (Yang et al., 2010) and polyphenol oxidases (Wu et al., 2010). Fraction 3 contained more non-protein components, including 18% carbohydrates, 10% polyphenols, and 40% undetermined components. As the molecular weights of the components in Fraction 3 were lower than 3 kDa , it can be speculated that these were mainly peptides, oligosaccharides, free polyphenols, organic acids, or lipids that could also be precipitated by acidic conditions.

3.1.2. Free polyphenols and oligosaccharides in the APE

To quantify and identify the free polyphenols and oligosaccharides, the APE was dialyzed with a membrane of 3 kDa MWCO. The polyphenol content in the dialysate was determined, and the molecules were

identified by LC-MS (Table 1), while the monosaccharide composition of the carbohydrates in the APE was determined by HPLC (Table 2).

Approximately 30% of total polyphenols were present in the dialysate, and nine types of polyphenols were identified, of which six were flavonoids (Table 1). Gallic acid, (+)-gallocatechin, epicatechin, epigallocatechin gallate, and (–)-epigallocatechin 3-(4-methyl-gallate) are typical tea polyphenols (Kerio et al., 2013), and these remained following alkaline extraction. Of these typical tea polyphenols, gallic acid, (+)-gallocatechin, and epicatechin contribute to the bitter taste of green tea (Hou et al., 2017), which may influence the taste of the APE. Other polyphenols might be generated during alkaline extraction. Caffeic acid 3-O-glucuronide, diosmetin 7-O-beta-D-glucuronopyranoside, and 8-hydroxyluteolin 8-glucoside 3'-sulfate could be the products of phenolic compounds and polysaccharides covalently linked with glycosidic bonds (Wang et al., 2019). 2-O-(4-hydroxycinnamoyl)-1-O-galloyl-beta-D-glucopyranoside could originate from lignin, which is degraded during alkaline extraction (Vanholme et al., 2010).

As listed in Table 2, the APE contained 18% carbohydrates, of which half constituted galacturonic acid mainly from homogalacturonan (HG) pectins (Caffall and Mohnen, 2009). After dialysis, 57 mg g^{-1} carbohydrates were removed, of which 37.2 mg g^{-1} was galacturonic acid, suggesting the degradation of HG pectin during alkaline extraction. Other major monosaccharides that were removed by dialysis were galactose and rhamnose. As arabinan, galactan, and arabinogalactan are the main branches of rhamnogalacturonan I (RGI) pectin, the remarkable loss of galactose during dialysis indicated that galactan was more vulnerable than the other branches of RGI pectin during the alkaline protein extraction step. After dialysis, only 2.2 mg g^{-1} of glucose was removed out of 16.3 mg g^{-1} glucose. It has already been shown that less than 5% cellulose could be extracted under our alkaline extraction conditions (Zhang et al., 2015), and the extracted cellulose fragments still possessed molecular weights larger than 3 kDa .

3.2. Enzymatic degradation of APE

3.2.1. Variation in APE components

Viscozyme® L was used to degrade carbohydrates, while pepsin and alkaline protease were used to partially degrade the proteins in the APE. The molecular weight distributions of the treated samples were determined by SEC, as illustrated in Fig. 2. Viscozyme® L and pepsin mainly degraded Fraction 2, while alkaline protease mainly degraded Fraction 1. Viscozyme® L degraded Fraction 2 due to the high carbohydrate content in Fraction 2. The differences in alkaline protease and pepsin on APE degradation might result from their hydrolysis conditions and their degradation mechanisms. As the pI of APE is about 3.6, proteins with large molecular weights could aggregate at pH 4 (Caffall and Mohnen, 2009) (see Supplementary Data Fig. S3), thus influencing the efficiency of pepsin hydrolysis (Mune Mune, 2015). Additionally, alkaline protease degrades peptide bonds with hydrophobic amino acids (Dee et al., 2011), while pepsin degrades peptide bonds with aromatic or acidic

Table 1
Identification of free phenols in APE by LC-MS.

Peak no.	TR (min)	Compound	Predicted MS m/z	observed MS m/z	Class
1	8.7	Gallic acid	171.0288	171.0288	Benzoic acids and derivatives
2	16.6	(+)-Gallocatechin	307.0812	307.0813	Flavonoids
3	20.98	Diosmetin 7-O-beta-D-glucuronopyranoside	459.0928	459.0926	Flavonoids
4	21.64	Caffeic acid 3-O-glucuronide	321.0616	321.0616	Organooxygen compounds (Phenolic glycosides)
5	22.42	Epicatechin	291.0863	291.0866	Flavonoids
6	23.56	Epigallocatechin gallate	459.0922	459.0922	Flavonoids
7	24.27	(–)-Epigallocatechin 3-(4-methyl-gallate)	473.1078	473.1078	Flavonoids
8	25.64	2-O-(4-Hydroxycinnamoyl)-1-O-galloyl-beta-D-glucopyranoside	443.0984	443.0986	Tannins
9	28.06	8-Hydroxyluteolin 8-glucoside 3'-sulfate	197.1170	197.1170	Flavonoids

Table 2
Variation in the monosaccharide composition of APE after dialysis (mg g⁻¹ dry matter).

	Arabinose	Xylose	Mannose	Glucose	Galactose	Rhamnose	Galacturonic acid	Total
Control	26.5 ± 1.7	10.0 ± 0.8	4.4 ± 0.3	16.3 ± 0.9	18.2 ± 0.8	15.2 ± 0.7	89.7 ± 0.5	180.3 ± 2.1
After dialysis	22.0 ± 0.4	6.2 ± 0.8	2.3 ± 0.8	14.1 ± 0.4	13.6 ± 1.2	12.3 ± 0.7	52.5 ± 1.2	123.1 ± 1.3

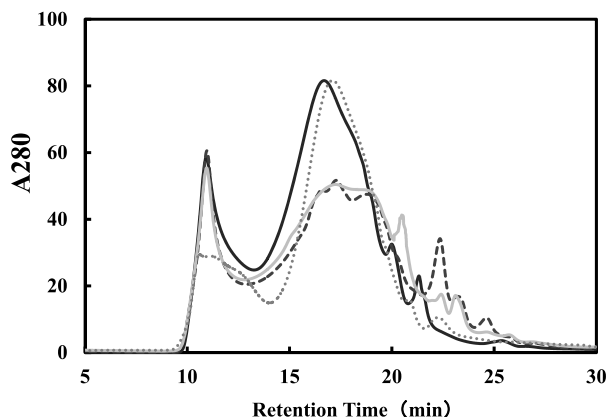


Fig. 2. The molecular weight change of alkaline protein extract from tea residue under different enzymatic hydrolysis conditions (Separation conditions: 0.05M, pH7 in PBS buffer, column model: SEC300, flow rate 0.5 mL min⁻¹, time 30min, 25 °C).fx10: Control; fx11: Viscozyme® L; fx12: Pepsin; fx13: Alkaline protease.

amino acids (Barrett et al., 2004). After a dialysis using membrane of 3 kDa MWCO, the content of protein in APE was not decreased (data not shown), suggesting that the degrading proteins in the APE were still large molecules. The peaks appeared between the retention time of 22–25 min attributed from non-protein components, which was not covalently linked to protein and was released after protein hydrolysis.

3.2.2. Analysis of APE hydrolysates

After enzyme degradation, the APE hydrolysates were dialyzed, and the amount of released polyphenols and oligosaccharides with molecular weights lower than 3 kDa were determined. The remaining monosaccharide and polyphenol contents in the APE hydrolysates are presented in Table 3.

Using enzymes to degrade carbohydrates or proteins in the APE could release polyphenols. Compared with the control, the amount of polyphenols in the dialysate decreased by 32% using Viscozyme® L, by 26% using pepsin, and by 11% using alkaline protease. As most polyphenols were in Fraction 2, the degradation of Fraction 2 components using Viscozyme® L or pepsin could release more free polyphenols that were noncovalently linked with proteins or carbohydrates. Some polyphenols that were covalently linked with carbohydrates by glycosidic bonds could be released to the dialysate due to the degradation of carbohydrates to small oligosaccharides. Polyphenols with covalently bound proteins would mostly be retained in the retention, as the degraded proteins still possessed molecular weights higher than 3 kDa.

Following treatment with protease, most monosaccharides remained in the retention, suggesting that only minor oligosaccharides bound with

Table 3
Contents of monosaccharides and polyphenols in the APE hydrolysate after dialysis using membrane with 3 kDa molecular weight cut-off (mg g⁻¹ dry matter).

Treatments	Polyphenol	Rhamnose	Galactose	Glucose	Mannose	Xylose	Arabinose	Galacturonic acid
Control*	22.5 ± 0.6 ^a	12.3 ± 0.7 ^a	13.6 ± 1.2 ^a	14.1 ± 0.4 ^a	2.3 ± 0.8 ^a	6.2 ± 0.8 ^a	22.0 ± 0.4 ^a	52.5 ± 1.2 ^a
Viscozyme® L	15.3 ± 1.7 ^c	8.2 ± 0.4 ^b	4.6 ± 0.7 ^b	3.6 ± 0.9 ^b	1.6 ± 0.4 ^a	4.9 ± 0.6 ^a	9.2 ± 0.3 ^c	9.5 ± 2.0 ^d
Pepsin	16.6 ± 0.6 ^c	12 ± 0.4 ^a	13.1 ± 1.1 ^a	13.3 ± 1.0 ^a	2.2 ± 0.8 ^a	5.2 ± 0.4 ^a	19.2 ± 1.2 ^b	41.6 ± 0.6 ^c
Alkaline protease	19.9 ± 1.6 ^b	11.7 ± 0.8 ^a	13.2 ± 1.2 ^a	13.6 ± 0.9 ^a	2.1 ± 0.3 ^a	5.9 ± 0.8 ^a	20.9 ± 1.6 ^{ab}	49.3 ± 1.3 ^b

Mean values in the same row with different letters behind the numbers are significantly different (one-way ANOVA, $p < 0.05$).

proteins could be released by protein degradation. Carbohydrates in Fraction 1 and Fraction 2 were mainly large polymers that could be degraded by Viscozyme® L. After Viscozyme® L degradation, more than 70% of the galacturonic acid, glucose, and galactose were in the dialysate, suggesting the fragments from HG pectin, cellulose, and RG I pectin were degraded (Caffall and Mohnen, 2009). Half of the rhamnose was retained in the retention after Viscozyme® L degradation, indicating that the RGII pectin was not hydrolyzed. More than 80% of xylose and mannose was found in the retention, suggesting that hemi-celluloses (including xylan, glucuronoxylan, and galactoglucomannans) could not be degraded by Viscozyme® L.

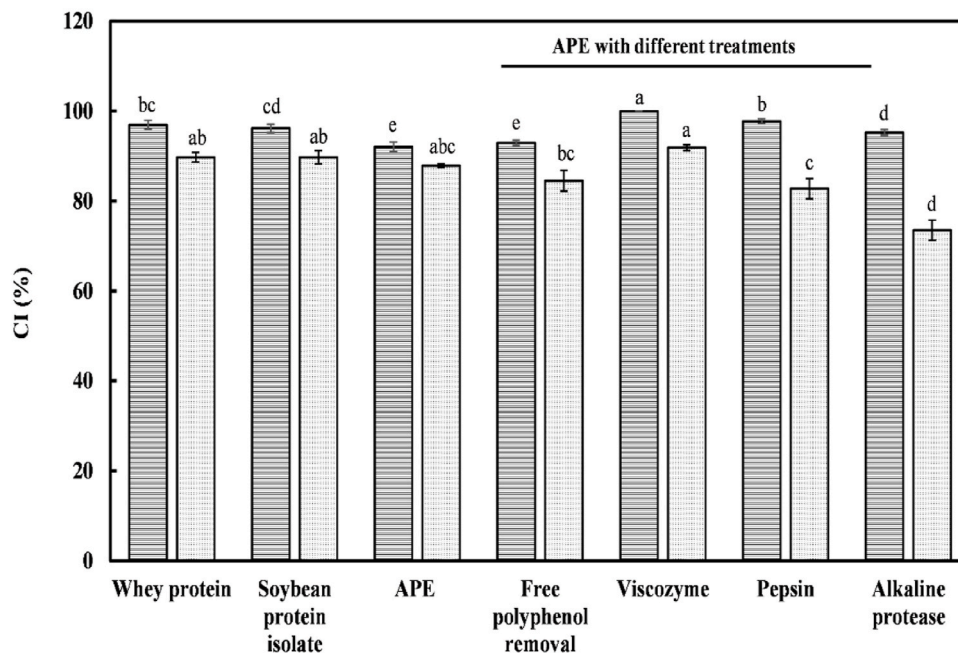
3.3. Influence of enzymatic degradation on the APE's emulsification properties

Emulsions with oil/water ratio of 7:3 using 1 mg mL⁻¹ whey protein, soybean protein isolate, and APEs (treated or untreated) as emulsifiers were made. After stood still for 10min or stored for 15 days at 25 °C, CI and EAI of each sample were determined, and the results were presented in Fig. 3a and Fig. 3b.

Based on the results, APE had similar CI and emulsification stability (see Fig. 3a) as those of whey protein and soybean isolate with a better EAI value (see Fig. 3b). After emulsification for 10 min, the CI of the APE was about 92%, which was only slightly lower than that of whey protein (97%) and soybean protein isolate (96%). In contrast, the EAI value of APE emulsion was about 44 m² g⁻¹, higher than those of whey protein (37 m² g⁻¹) and soybean protein isolate (35 m² g⁻¹). After stored for 15 days, the CI of the APE emulsion decreased to 88%, and its EAI value remained 44 m² g⁻¹, suggesting APE had very good emulsification stability.

To further improve the emulsification properties of APE, degradation of carbohydrates in APE was proven as a better way than removal of polyphenol or protein degradation. As shown in Figs. 3 and 1g Viscozyme® L treated APE had a CI value of 100% with EAI value of 74 m² g⁻¹, and the numbers only slightly reduced to 92% and 67 m² g⁻¹. Polyphenol removal as well as protein degradation using alkaline protease did not improve the emulsification properties of APE. The emulsion stability of alkaline protease treated APE was even decreased that its CI value decreased from 95% to 74% after storing for 15 days. Pepsin could slightly improve the emulsification properties of APE that the EAI value of emulsion increased to 53 m² g⁻¹. As pepsin and Viscozyme® L both targeted on components in Fraction 2, a fraction containing most HG pectin, it can be deduced that the emulsification properties of APE were inhibited by carbohydrates, in which HG pectin was the main components. Although HG pectin was proven having emulsification properties (Mendez et al., 2021), the inhibition of carbohydrate might result from the formation of protein-pectin complex (Ngouémazong et al., 2015), by which forming depletion flocculation (Verkempinck et al., 2018) and thus reducing its emulsification properties.

a.



b.

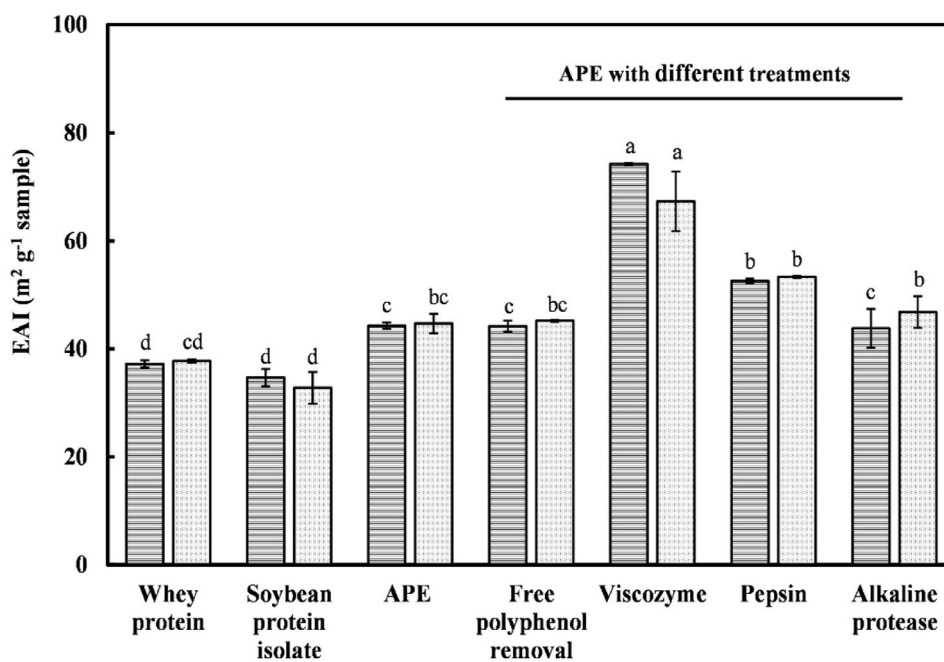


Fig. 3. Creaming index (CI, %, a) and emulsification activity index value (EAI, $\text{m}^2 \text{g}^{-1}$, b) of emulsions obtained using different emulsifiers in a 7:3 of oil/water at 25 °C. fx14: Emulsion stood still for 10 min; fx15: Emulsion stored for 15 days.

Prospectively, as the fractions in the APE exhibited great differences in molecular weights, study on interaction of these molecules in APE can offer the basis for its emulsification properties. In fact, these fractions can be separated by ultrafiltration, which can be easily adapted to the integrated process for leafy biomass (Zhang et al., 2016a). A membrane with a molecular weight cutoff of 100 kDa can be used, and thus a protein fraction (Fraction 1) with a purity higher than 80% can be

obtained. This fraction with high protein content may have better emulsification properties leading to higher market value. In addition, a prior process to obtain pectin product in the integrated process can also improve the emulsification properties of protein extract. This integrated process can be adapted to other leaf biomass (Zhang et al., 2014), showing more possibilities for leaf protein as emulsifiers.

4. Conclusion

The APE contained 52% protein, 18% carbohydrates, and 3% polyphenols. The APE had similar CI and emulsification stability as those of whey protein and soybean isolate with a better EAI value of $44 \text{ m}^2 \text{ g}^{-1}$. The emulsification properties of the APE can be further improved using Viscozyme® L indicating protein played the main role in emulsification that was inhibited by carbohydrates, mainly containing HG pectin. Based on the molecular weights, APE can be divided into three fractions, in which a fraction had a content of 83% with molecular weights higher than 100 kDa. Purification of APE to obtain high protein content may further improve its emulsification properties.

CRedit authorship contribution statement

Zexin Lin: Investigation, Writing – original draft, Writing – review & editing. **Han Wei:** Validation, Formal analysis. **Yufei Zhang:** Validation. **Pai Liu:** Methodology, Validation. **Yongxue Liu:** Writing – original draft. **Zhensheng Huang:** Funding acquisition. **Xucong Lv:** Writing – review & editing. **Yanyan Zhang:** Writing – review & editing. **Chen Zhang:** Conceptualization, 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.

Acknowledgements

This work was supported by the Industrial technology development and application plan guided projects of Fujian Province (No. 2018N0013) and the NSFC (No. 31701649).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crfs.2022.07.016>.

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