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Analytical Methods

Studies on tea protein extraction using alkaline and enzyme methods

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Abstract

The extraction of proteins from tea leave pulps, using alkaline and enzyme methods, were investigated in this work. Altogether four enzymes (neutrase, alcalase, protamex and flavourzyme) were examined for tea protein extraction. It was found that an alkaline method produced a high protein yield (56.4% extraction rate). The use of an enzyme alone appeared to be less effective in extracting tea protein (less than 20% extraction rate). However, a combination of two enzymes (for example, alcalase and protamex at a 1:3 weight ratio) became much more capable in extracting protein and led to a much higher extraction rate (as high as 47.8%). Various extraction conditions, such as volume–weight ratio between the extraction solution and the weight of dry tea leave pulps (V/W), the extraction time (t), pH, temperature (T), agent concentration (c) have been investigated for their influences. Optimum combinations of extraction conditions were obtained using the orthogonal analysis technique. The extracted tea protein was further purified for the analysis of molecular weight distribution and amino acid compositions. It was found that tea protein was composed of at least seven different types of proteins and had amino acid composition more or less similar to that of soy protein.

Keywords: Tea protein; Protein extraction; Alkaline extraction; Enzyme extraction; Alcalase; Protamex; Neutrase; Flavourzyme

1. Introduction

Protein molecules tend to unfold and even become fully denatured under unfavoured conditions, such as a high temperature, an acidified condition, a high pressure or even excessive shear. Denatured protein molecules will aggregate and/or crosslink to form larger clusters and, at high concentrations, will form a three-dimensional solid-like network (or a gel). Therefore, proteins are regarded as one of the main classes of building blocks used in many semi-solid foods for conferring mechanical properties (Dickinson, 1997). Proteins are also recognised as one of the main classes of surface-active agents in liquid foods for stabilising dispersed particles and fat droplets (Dalgleish, 1997), due to the polarised distribution of hydrophobic and hydrophilic groups along the back bone. Protein molecules adsorb at the oil–water interface to lower the interfacial tension and, therefore, make such thermodynamically less favourable dispersed systems stable for an extended shelf life. The importance of protein application in foods can also be seen in many other aspects. For example, it was reported that, in combination with polysaccharides and starches, proteins could be applied as a meat alternative, as a fat replacer or a filler in manufacturing healthier foods (Roger, 2001). Proteins also have special uses as foaming agents or as functional ingredients for nutrient delivery in foods (Chen, Remondetto, & Subirade, 2006; Doi, 1993; Hettiarachchy & Ziegler, 1994).

Huge efforts have been made in extracting proteins from various sources for food applications. So far, proteins from two major sources (milk proteins and soy protein) are most widely used in the food industry, either as a general nutrients supply or as functional ingredients. Milk proteins (e.g., whey proteins or caseins) are probably the most commonly used proteins in all major types of food products (Fox &

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McSweeney, 1998). As from non-animal source, soy protein becomes increasingly used in food products because of its health benefits and characteristic physico-chemical properties (Endres, 2001; Hoogenkamp, 2005). Proteins from other sources have also been studied for their functionality and potential food applications. Examples include corn protein (Myers, Hojillaevangelista, & Johnson, 1994), wheat protein (Hettiarachchy, Griffin, & Gnanasambandam, 1996), rice protein (Morita, Ohhashi, Kasaoka, Ikai, & Kiriyama, 1996), seaweed protein (Fleurence, 1999), protein from yeast (Ganeva & Galutzov, 1999), fish protein (Afonso, Ferrer, & Borquez, 2004), etc. However, the applications of these proteins were still very limited, either due to the limited source of supply or the non-satisfactory functionality.

Tea is one of the most popular soft drinks in the world with an estimated total production of 3.2 million ton (year 2004). In China alone, the annual production of tea was close to a million ton (year 2004) and is increasing by 8.7% per annum. In the tea industry, high quality tea leaves were selected for the production of dried green tea or fermented tea, while low quality tea leaves would be used for the production of instant tea or tea drinks and for the extraction of tea polyphenols. Each year a large quantity of tea pulp from tea manufacturing processes is disposed, without further treatment, which is not only a waste of a natural resource but also becomes a concern to the environment. Researches show that tea leaves contain around 21–28% protein on dry-base (Gu, Lu, & Ye, 2002). It was recently reported that tea protein has the capability in protecting biological cells against mutagenesis caused by irradiation (Li et al., 2001). It is, therefore, expected that tea protein has a great potential as a functional ingredient for food applications. However, little has been done on the extraction of tea protein and there has been, so far, no established method for industrial applications.

The main aim of this work was to investigate the feasibility of using alkaline and enzyme methods for protein extraction from tea pulps. The use of alkaline solutions has already been widely used as an effective and feasible method for protein extraction from plant sources. Proteins in plant cells are hardly water soluble because of their hydrophobic nature and the disulphide bonding between protein molecules. It is believed that the high alkaline concentration helps to break down the hydrogen bonds and to dissociate hydrogen from carbolic, sulphate groups. The increased surface charge of protein molecules will then lead to an enhanced solubility in water. This has been proved by Guo, Pang, and Wang (2005), who used a 0.1 M NaOH solution and achieved 55% protein extraction from rice. Separate research even reported a 90% extraction of rice protein using a similar alkali approach (Sun & Tian, 2003). Enzyme is another effective agent for protein extraction. Protease and celluase had been used to extract protein from soybean and it was found that protease produced a significantly higher extraction yield (Rosenthal, Pyle, Niranjan, Gilmour, & Trinca, 2001). Examples of enzyme extraction of protein can also be seen from the works by Tang, Hettiarachchy, and Shellhammer (2002) Hanmoungjai, Pyle, and Niranjan (2002). Many other methods have also been investigated for protein extraction. For example, Wen and Luthe (1985) used urea for the extraction of rice protein. Seung and Lee (1999) used surface-active agents (sodium stearate and sodium dodecyl sulphonate) to extract rice protein but found they were less effective than a sodium hydroxide solution (0.2%). Ganeva and Galutzov (1999) showed that the use of high intensity electric pulses could provoke considerable release of protein from yeast.

In this study, we used alkaline and enzyme methods for the extraction of protein from tea pulps. Four enzymes (neutrase, alcalase, protamex, and flavourzyme) were used for the investigation. The choice of the four enzymes was made based on the fact that these enzymes have already been widely used in the food industry for various applications and are proved to be safe and that high grade samples were commercially available, requiring no further treatment. The extraction yield of tea protein was examined for each method and the effects of various extraction conditions, including agent concentration (alkali, enzyme), extraction time, volume–weight ratio between solvent and tea leave pulps, extraction temperature, and pH have also been investigated. The orthogonal analysis method was used to obtain an optimum combination of extraction conditions so that a maximum rate of protein extraction could be achieved.

2. Materials and methods

Dried green tea leaves (Longjing brand), a product manufactured in the West-Lake region in Hangzhou (China), was used for protein extraction. Sodium hydroxide (NaOH) was purchased from Hangzhou Xiaoshan Chemicals (Hangzhou, China). Sodium chloride (NaCl) was purchased from Shanghai General Regents (Shanghai, China). Sulphuric acid (H_2SO_4) was purchased from Shanghai No. 1 Reagents (Shanghai, China). Hydrochloric acid (HCl) and boric acid (H_3BO_3) were purchased from Taichang No. 2 Chemicals (Jiangsu, China). Potassium sulphate (K_2SO_4) and cupric sulphate ($CuSO_4$) were supplied by Hangzhou Huipu Instruments Ltd. (Hangzhou, China). Ethanol (C_2H_5OH) was obtained from Hangzhou Chemicals Ltd. (Hangzhou, China). All chemicals used in this work were AR grade. Four different enzymes, neutrase, alcalase, protamex and flavourzyme, were supplied by Danmano and Norde BioReagents Ltd. (Jiangsu, China).

2.1. Preparation of tea leaf pulps

A weighted amount of dried green tea leaves were put into a glass container and topped with pre-measured amount (1200 ml) of boiling water. After 15 min of soaking, the water was filtered out and the same amount of boiling water was added again to the container. This soaking

process was repeated three times. Tea leaves were then dried in a fanned electric oven (Model 101A-2, Shanghai General Experimental Instruments, Shanghai) at 100 °C for 4 h before been mashed using a multi-function food processor (Model SQ211-9B, Shanghai Shuaijia Electronic Scientific Ltd., Shanghai). The mashed tea leaves were then passed through a sieve of 60 μm and ready for extraction.

2.2. Alkaline extraction of tea protein

For alkali extraction of tea protein, mashed tea leaves were mixed with a sodium hydroxide solution and stirred continuously for a set time and at a controlled temperature using a vibrator (Model SHZ-82 Vibrator, Suzhou Guohua Electronic Ltd., Jiangsu, China). The mixture was then centrifuged at 2000 rpm for 10 min using a TDL-40B Centrifuge (Beijing Yali Scientific Instruments, Beijing). The supernatant was carefully collected for further treatment.

2.3. Enzyme extraction of tea protein

Mashed tea leave pulps were weighed into a glass container containing a known amount of pH-adjusted and temperature-controlled water. Enzyme was added while it was gently stirred using the SHZ-82 Vibrator. The mixture was kept stirred for a set length of time before being centrifuged. The supernatant phase was carefully collected for further treatment.

2.4. Purification of tea protein

The procedure for tea protein purification is illustrated in Fig. 1. The collected raw protein solution from either alkaline extraction or enzyme extraction was added with ammonia sulphate [$(NH_4)_2SO_4$] till it reached its saturation. The solution was then left still for the precipitation of protein. The solution was centrifuged and the collected protein precipitate was washed with acetone (for discolouration), at a ratio of 1:1 volume to weight and stirred for 30 min before being filtrated. This procedure was repeated four times. The protein was then dialysed against distilled water, which was repeatedly changed for every 2 h until the conductivity of the water phase reached a constant minimum.

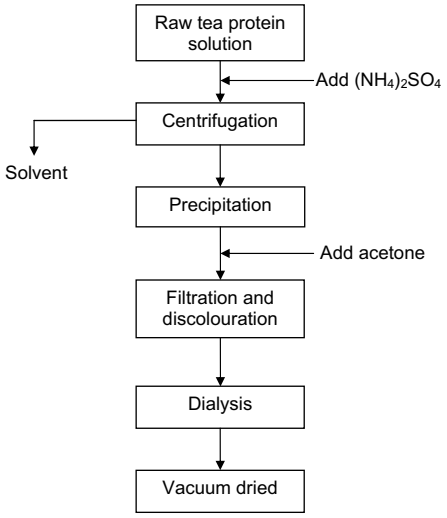

Fig. 1. The purification procedure of tea protein.

The amount of protein was quantified using the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as the standard. The extraction method was assessed by the protein yield or the extraction rate, which was defined as the percentage ratio of the protein quantity extracted to the total amount of the protein in the tea leaf. A discontinuous SDS gel electrophoresis of 15% acrylamide was performed using a vertical mini-gel system of 0.75 mm thickness. The gel was prepared according to the method described by Laemmli (1970). Proteins of different molecular weights, β-lactoglobulin (18.4 kDa), lactate dehydrogenase (35.0 kDa), ovalbumin (45.0 kDa), bovine serum albumin (66.2 kDa) and galactosidase (116.0 kDa), were used as markers. The molecular weight of a tea protein was determined by comparing its migration distance with the standard markers. The amino acid analysis of tea protein was performed using a Hitachi 835-50 Amino Acid Analyser (Hitachi, Japan).

3. Results and discussion

3.1. Tea protein extraction by alkaline method

The use of alkali solutions has been widely recognised as a feasible method for protein extraction from plant sources. However, its effectiveness depends highly on the extraction conditions such as, agent concentration, extraction temperature, extraction time, volume–weight ratio between the extraction solvent and the raw material (Guo et al., 2005; Sun & Tian, 2003). In this study, NaOH was used as the sole extraction agent and our main focus was to see how protein extraction was influenced by various conditions. Fig. 2 shows the tea protein extraction rate as a function of various operation parameters. It is for sure that alkaline extraction is applicable to extract protein from tea pulps. However, the extraction yield varied significantly when different conditions were applied. For the effect of alkali concentration, extraction experiments were carried out at a fixed extraction time (2 h), a constant temperature (30 °C) and a constant volume–weight ratio (35:1). It was found that, at relatively low alkali concentrations (<0.08 M NaOH), the extraction rate increased almost linearly with the increase of alkali concentration. However, further increase of alkali concentration appeared to be less impressive. Only a marginal change of the extraction rate was observed when the alkali concentration was increased from 0.08 M to 0.14 M (Fig. 2a).

The effect of temperature on tea protein extraction was examined for a fixed extraction time (2 h), a fixed alkali concentration (0.06 M NaOH) and a fixed volume–weight

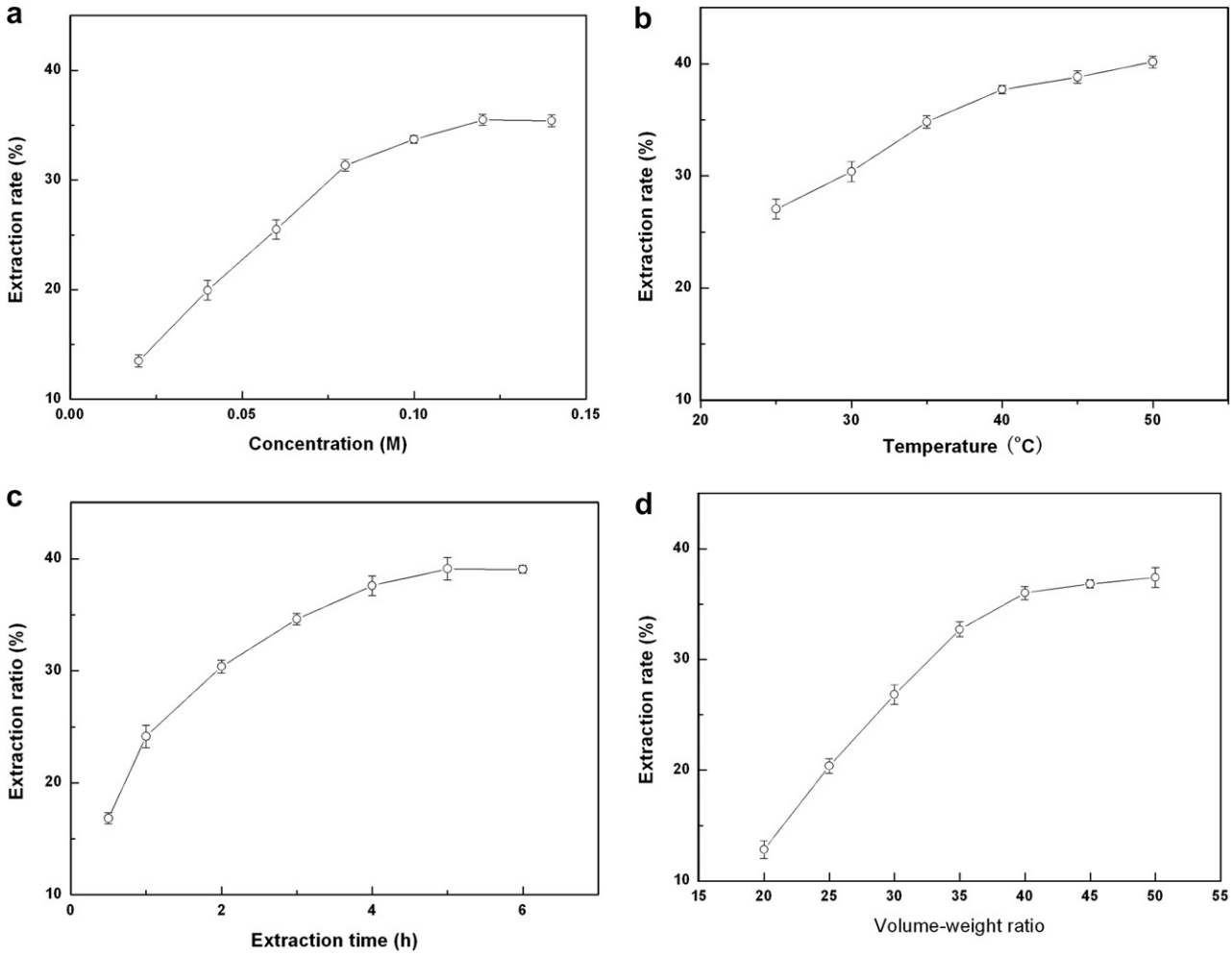

Fig. 2. Effects of various factors on the tea protein extraction using the alkaline method: (a) effect of alkali concentration; (b) effect of extraction temperature; (c) effect of extraction time and (d) effect of volume–weight ratio of solvent and dry tea leave pulps.

ratio (35:1). Generally speaking, a higher temperature would be beneficial for tea protein extraction (Fig. 2b). The most significant effect was observed between 25 and 40 °C, where the extraction rate increased from 27% at 25 °C to around 38% at 40 °C, almost one percentage increase for each Celsius degree increase. However, temperature change seemed to become less important above 40 °C. A further increase of 10 °C (from 40 °C to 50 °C) only led to a slight increase of extraction rate (from around 38% at 40 °C to less than 41% at 50 °C). Therefore, for both technical and economical reasons, 40 °C could be a suitable choice for tea protein extraction.

The effect of extraction time on protein yield was shown in Fig. 2c (at fixed contraction conditions: 30 °C, 0.06 M NaOH, and volume–weight ratio 35:1). It was found that a longer extraction time was generally beneficial. The most dramatic effect of the extraction time was observed at the first two hours of extraction, where the protein yield increased sharply with the time. It appeared that the time effect faded gradually after 4 h and there was only a marginal increase of protein yield between the 4 h extraction and the 6 h extraction. Therefore, a time between 3 and 4 h could be most suitable for tea protein extraction. In addition to alkali concentration, temperature and extraction time, the volume–weight ratio between the extraction solvent and the mass of tea pulps was also proved to play an important role in tea protein extraction. As shown in Fig. 2d, the extraction rate increased almost linearly with the volume–weight ratio to up to a ratio of 40:1, but remained little changed at higher volume–weight ratios. A higher solvent volume would mean a lower protein concentration in the solvent. This will give a larger protein concentration difference between that in the solid phase and that in the solvent and, therefore, a greater driving force for mass diffusion during the extraction process. However, this beneficial effect will diminish gradually when a very large volume of solvent is used and will be outweighed by the difficultly of having to handle the extra volume of solvent. Therefore, a volume–weight ratio of 40:1 seems to be suitable for tea protein extraction using the alkaline method.

The above four extraction parameters have also been analysed at three different levels using the orthogonal method, for their relative importance in influencing protein

yield and for their optimum combination. Altogether nine experiments, involving different extraction conditions, were conducted. Table 1 shows the extraction yield for each experiment, where k is the average extraction rate of the parameter at a chosen level and R is the maximum difference between the three k values. A higher k value would indicate a preferred level for the chosen parameter, while a higher R value would indicate a greater influence of the parameter. Based on this principle, optimum extraction conditions for each operation parameter can be determined from the maximum yield value of k_1, k_2 and k_3 of each column. For example, the optimum alkali concentration should be at level 3 (0.10 M), where k_3 gives the maximum yield value (50.6%). Similarly, we can determine that the optimum volume–weight ratio should be 40:1, the optimum extraction time should be 5 h and the optimum extraction temperature should be 40 °C. This leads to an optimal combination of extraction conditions. It was further confirmed that using such a combination, as much as 56.4% tea protein could be obtained, a yield comparable to that for rice protein extraction (55%) using a 0.1 M NaOH (Guo et al., 2005). The orthogonal analysis also revealed the relative importance of each factor in affecting tea protein extraction. As shown in Table 1, with an R value of 8.2, the extraction time seems to be the most critical factor, followed by the alkali concentration (R value 4.1). With an R value of only 0.8, the variation of volume–weight ratio appears to have the least influence in tea protein extraction.

3.2. Tea protein extraction by enzyme methods

Different enzymes may have different capabilities in extracting proteins because of their characteristic nature, as has been observed by many other researchers (Wang, Qiu, & Fan, 2002). In this work, four proteolytic enzymes (neutrase, alcalase, protamex and flavourzyme) were examined for their capability in extracting tea protein, used either as an individual or as a mixture. Table 2 shows the extraction conditions for the four enzymes, where different temperature and pH were used according to supplier's recommendation but the enzyme concentration, volume–weight ratio, and extraction time remained the same for all four enzymes. Alcalase and protamex out-performed the other two enzymes in terms of protein yield, with an extraction rate of 18.3% and 11.0%, respectively. The protein yields were less satisfactory for the other two enzymes: neutrase extracted 9.5% tea protein and flavourzyme only managed to extract 7.8% tea protein. Therefore, alcalase and protamex were chosen for further studies of their extraction conditions.

Fig. 3 shows the effects of enzyme concentration, volume–weight ratio and the extraction time on tea protein extraction for the two enzymes, where open cycle represents the alcalase enzyme and solid cycle represents the protamex enzyme. The extraction was carried out at the controlled pH condition and temperature as shown in Table 2. It can be seen from Fig. 3a that the alcalase concentration has a significant influence on tea protein extraction (at volume–weight ratio 30:1 and 2 h extraction time). It appeared that too low or too high alcalase concentration was not preferable. The highest yield of protein extraction was observed at a concentration of 4% alcalase. Similar behaviour was also observed for the protamex enzyme. Even though this enzyme appeared to have a much lower protein yield than alcalase, a maximum extraction rate was also found at 4% concentration. The maximum protein yield at a medium enzyme concentration indicates the changing role of the enzyme at higher concentrations. There is no doubt that the increase of enzyme concentration would normally enhance the solubility of protein into the solvent and, therefore, increase the extraction yield. However, the existence of free enzyme in the solvent could interact with the protein and cause degradation of protein molecules. Therefore, there should be an optimum enzyme concentration for a maximum protein yield. Another possible explanation of the maximum protein yield could be due to the so called competitive inhibition. The extracted protein molecules function as the inhibitor combining with the enzyme to form a complex and thereby preventing its activity

Table 1
Orthogonal analysis of the alkaline extraction method

Number	[NaOH] (M)	V/W	Time (h)	T (°C)	Extraction rate (%)
Level 1	0.06	35:1	3	35	
Level 2	0.08	40:1	4	40	
Level 3	0.10	45:1	5	45	
Experiment 1	0.06	35:1	3	35	41.2
Experiment 2	0.06	40:1	4	40	47.5
Experiment 3	0.06	45:1	5	45	50.9
Experiment 4	0.08	35:1	4	45	48.3
Experiment 5	0.08	40:1	5	35	52.7
Experiment 6	0.08	45:1	3	40	46.2
Experiment 7	0.10	35:1	5	40	55.4
Experiment 8	0.10	40:1	3	45	47.1
Experiment 9	0.10	45:1	4	35	49.4
k_1	46.5	48.3	44.8	47.8	
k_2	49.1	49.1	48.4	49.7	
k_3	50.6	48.8	53.0	48.8	
R	4.1	0.8	8.2	1.9	

Nine experiments were designed to cover four factors at three different levels.

Table 2
Comparison of tea protein extraction using different enzymes

Enzyme	Temperature (°C)	pH	Enzyme concentration (%)	V/W	Time (h)	Extraction rate (%)
Neutrase	50	7.0	2	30:1	2	8.5
Alcalase	55	7.5	2	30:1	2	18.3
Protamex	55	6.5	2	30:1	2	11.0
Flavourzyme	50	6.0	2	30:1	2	7.8

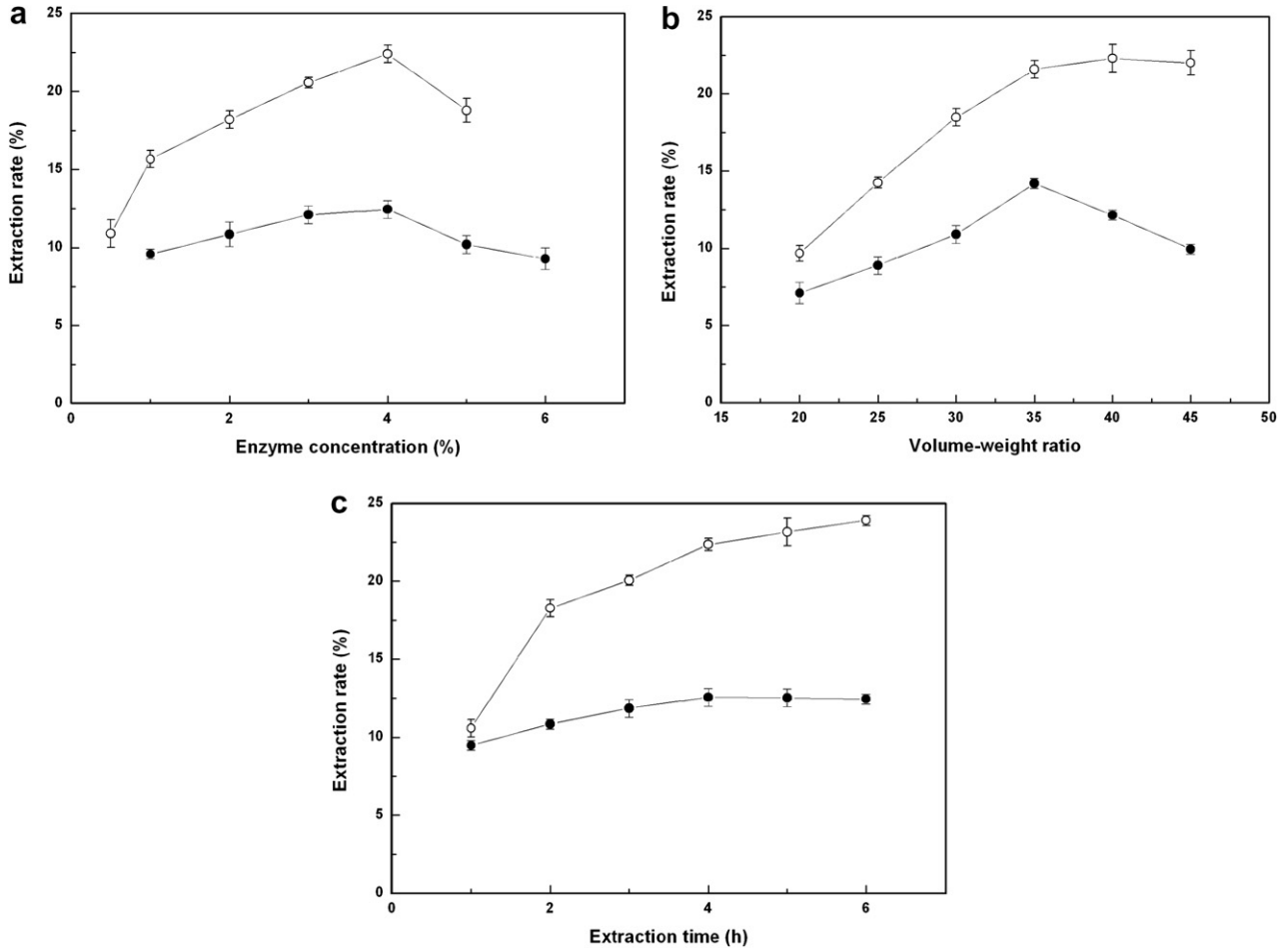

Fig. 3. Effects of various factors on the tea protein extraction using enzyme methods: (a) effect of enzyme concentration; (b) effect of volume–weight ratio between solvent and dry tea leave pulps and (c) effect of extraction time (○: alcalase enzyme; ●: protamex enzyme).

(Laidler & Bunting, 1973, chap. 3). Even though there is no evidence yet which of the two mechanisms is the main cause here, it is known that either too low or too high an enzyme concentration is not desirable for protein extraction.

The optimum volume–weight ratio of the enzyme solution and dry tea leave pulps was tested over the range between 20:1 and 45:1 (at an enzyme concentration 2% and 2 h extraction time) and the results are shown in Fig. 3b. At low volume–weight ratios (<35:1), the protein extraction rate showed almost a linear increase for both enzymes but further increase of volume–weight ratios (>35:1) showed different patterns to the two enzymes. The extraction rate started to level off for the alcalase enzyme and started to decrease for the protamex enzyme. One may speculate that the diluting effect of high solvent volume could play a role in this case but it is difficult to explain why higher volume–weight ratios would cause a decreased rate of protein extraction for the protamex enzyme. A possible explanation would be the protein degradation due to the existence of the free protamex enzyme. But, at only 2% enzyme concentration, the existence of extra protamex enzyme seems doubtful.

The extraction time seems to have a positive role for both enzymes over the investigated extraction length (up to 6 h) (Fig. 3c). Compared to protamex, the alcalase enzyme seems to have a much increased protein yield with the extended extraction length. For alcalase, the most dramatic effect of extraction time was observed at the first two hours. After 4 h, the effect of extraction time became less significant. For protamex, the overall extraction time appeared to be much less influential. The protein extraction rate increased at the first 3 h but hardly changed thereafter. This may indicate the fast decaying activity of this enzyme.

Orthogonal analysis has been carried out for both the alcalase method and the protamex method. The above three factors (volume–weight ratio, enzyme concentration and the extraction time) were tested at three different levels. For alcalase, it was found that a volume–weight ratio of 35:1, an enzyme concentration of 4% and an extraction time of 4 h gave an optimal combination (see Table 3). Further experiments confirmed that under these conditions, the alcalase method could lead to an extraction of 34.2% tea protein. Of the three parameters, the enzyme concentration ($R = 4.2$) and the volume–weight ratio ($R = 3.6$) have a more dominant influence on tea protein extraction. With

Table 3
Orthogonal analysis for the alcalase extraction method

	V/W	Enzyme concentration (%)	Extraction time (h)	Extraction rate (%)
Level 1	30:1	3	3	
Level 2	35:1	4	4	
Level 3	40:1	5	5	
Experiment 1	30:1	3	3	24.4
Experiment 2	30:1	4	4	30.6
Experiment 3	30:1	5	5	28.4
Experiment 4	35:1	3	5	30.6
Experiment 5	35:1	4	4	33.9
Experiment 6	35:1	5	3	29.9
Experiment 7	40:1	3	5	27.3
Experiment 8	40:1	4	3	30.3
Experiment 9	40:1	5	4	28.7
k_1	27.8	27.4	28.2	
k_2	31.4	31.6	29.9	
k_3	28.7	29.0	29.8	
R	3.6	4.2	1.7	

Nine experiments were designed to cover three factors at three different levels.

a smaller R value (1.7), the extraction time appears to be relatively less critical. For the protamex method, the optimum extraction condition would be a volume–weight ratio of 35:1, an enzyme concentration of 3% and an extraction time of 4 h (see Table 4). Orthogonal analysis also showed that all three factors played an important role in influencing protamex performance in tea protein extraction. It appeared that the volume–weight ratio had the most significant influence ($R = 3.2$), while extraction time had a relatively minor influence ($R = 1.9$).

The effect of pH variation and temperature change on tea protein extraction, using alcalase and protamex methods, had also been investigated and their effects were found to be very significant. As can be seen from Fig. 4, both enzymes have an optimal performance at a certain pH condition. Alcalase had the highest extraction rate at pH 8.0, while protamex extracted most at a slightly lower pH (7.5). We noticed that the optimum pH for both enzymes were higher than the values recommended by the supplier (see Table 2). Temperature is another important factor which could have a dramatic effect on enzyme activities. A temperature which is too high or too low would either kill or inactivate enzymes. Therefore, only a small temperature variation (from 45 °C to 65 °C) was investigated. Over this small range, temperature seemed to have a rather limited effect on the enzyme's capability in extracting tea protein. However, an optimum temperature was still identifiable for both enzymes. Alcalase achieved the highest protein yield at around 60 °C and protamex had its best performance at 55 °C (see Fig. 5).

Applying the optimum pH and temperature condition together with those obtained from orthogonal analysis (see Tables 3 and 4), we found that the extraction rate of tea protein could be increased significantly for both enzymes. An extraction rate as high as 41.9% of tea protein was obtained for the alcalase method (volume–weight ratio 35:1, enzyme concentration 4%, extraction time, 4 h, pH 8.0 and temperature 60 °C), while a maximum of 24.4% tea protein was extracted using the protamex method (volume–weight ratio 35:1, enzyme concentration 3%, extraction time 4 h, pH 7.5, and temperature 55 °C), a significant improvement compared to the rates shown in Fig. 3.

Since the two enzymes differ from each other and behave differently at different conditions, it is worth investigation whether there is any synergetic effect, if the two enzymes are applied together, in extracting tea protein. The combined effect of the alcalase and protamex mixture was examined at different ratios (1:4, 1:3, 1:2, 1:1, 2:1, 3:1 and

Table 4
Orthogonal analysis for the protamex extraction method

	V/W	Enzyme concentration (%)	Extraction time (h)	Extraction rate (%)
Level 1	30:1	2	2	
Level 2	35:1	3	3	
Level 3	40:1	4	4	
Experiment 1	30:1	2	2	10.9
Experiment 2	30:1	3	3	14.3
Experiment 3	30:1	4	4	15.2
Experiment 4	35:1	2	3	14.8
Experiment 5	35;1	3	4	18.6
Experiment 6	35:1	4	2	16.3
Experiment 7	40:1	2	4	14.7
Experiment 8	40:1	3	2	15.6
Experiment 9	40:1	4	3	16.9
k_1	13.4	13.4	14.3	
k_2	16.6	16.2	15.3	
k_3	15.7	16.1	16.2	
R	3.2	2.8	1.9	

Nine experiments were designed to cover three factors at three different levels.

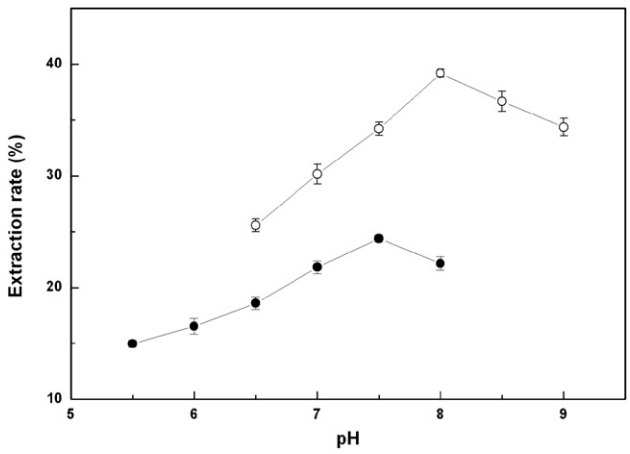

Fig. 4. Effects of pH on the tea protein extraction using enzyme methods (○: 4% alcalase enzyme, 35:1 volume–weight ratio, 4 h extraction time, 55 °C; ●: 3% protamex enzyme, 35:1 volume–weight ratio, 4 h extraction time, 55 °C).

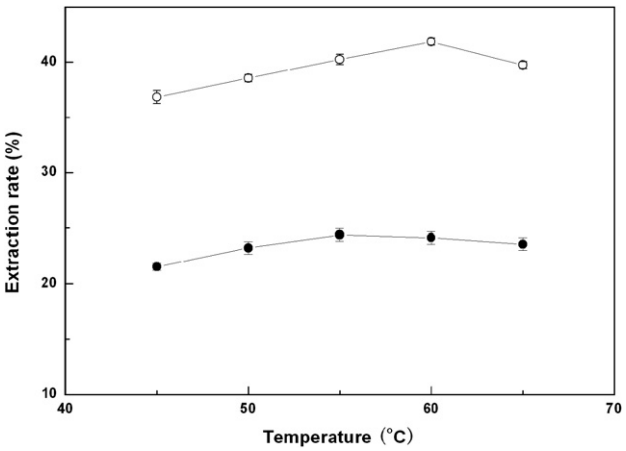

Fig. 5. Effects of temperature on the tea protein extraction using enzyme methods (○: 4% alcalase enzyme, 35:1 volume–weight ratio, 4 h extraction time, pH 8.0; ●: 3% protamex enzyme, 35:1 volume–weight ratio, 4 h extraction time, pH 7.5).

4:1) and results are shown in Fig. 6, where the results of pure protamex and alcalase are also shown for comparison. It was found that the presence of a second enzyme showed a significant enhancing effect of protein extraction. All combined alcalase and protamex systems produced much higher protein yield than that of either enzymes used alone. The most dramatic effect was observed when a quarter of the enzyme was replaced by the second one (at ratios of 1:3 or 3:1). For example, a replacement of 25% protamex with alcalase led to an almost 70% increase of the extraction rate (from only 25% for pure protamex to 42.1% for mixed alcalase + protamex), while the same amount of replacement of alcalase by protamex led to almost 18% increase of the extraction rate.

The mixed enzyme system (alcalase and protamex at 1:3) was further examined for its capability at different conditions using the orthogonal analysis. Altogether three factors

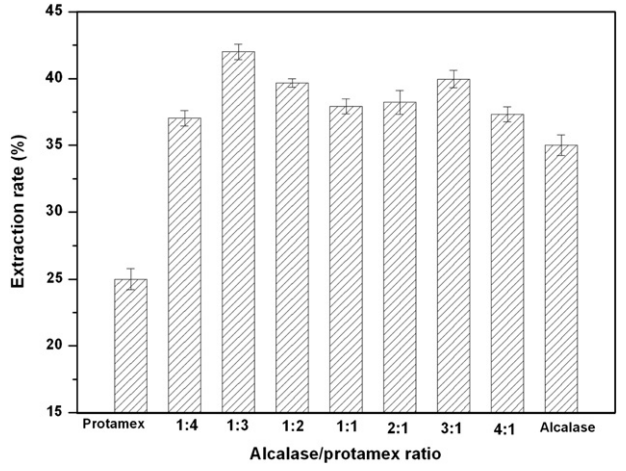

Fig. 6. Effects of mixed alcalase and protamex on tea protein extraction. The extraction was carried out at various fraction ratios between alcalase and protamex but with a constant total enzyme concentration 4%, 35:1 volume–weight ratio, 4 h extraction time, pH 7.5 and 55 °C.

(pH, temperature and total enzyme concentration) at three different levels were examined (see Table 5). The tests were carried out at a constant volume–weight ratio (35:1) and a constant extraction time (4 h) and showed that a combination of pH 8.0, 60 °C and 4% enzyme concentration gave the highest extraction yield. Of the three parameters, the total enzyme concentration appeared to have the least influence ($R = 1.3$). The pH ($R = 7.7$) and temperature ($R = 4.2$) seemed to have strong effects on the performance of combined enzymes. Further experiment showed that, under these conditions (a total enzyme concentration of 4%, a volume–weight ratio of 35:1, an extraction time of 4 h, pH 8.0, temperature 60 °C), an extraction rate of 47.8% could be obtained. Although alcalase only counts for a small fraction of the combined system (a quarter), its role seems to be critical in protein extraction. This can be seen from the fact that the optimal pH (8.0) and temperature (60 °C) conditions for combined systems are actually the optimal values when the alcalase is applied alone (see Figs. 4 and 5).

From the above results, it is clear that an alkaline method for tea protein extraction has the advantages of operating at a lower temperature (40 °C) and a high protein yield (up to 56.4%). However, a significant drawback of this method is the high alkali concentration, which would require efforts of water treatment afterwards and may become an environmental concern. High alkali concentration could also cause severe surface corrosion of equipments and devices. In addition, high alkali concentration may enhance the Maillard reaction of the extracted protein and lead to dark-brown-coloured products. Although enzyme methods for tea protein extraction operate at a relatively higher temperature (60 °C) and have a slightly lower extraction rate (up to 47.8%), it has the advantage of a mild pH condition (pH 8.0). The optimal volume–weight ratio is

Table 5
Orthogonal analysis for tea protein extraction using combined alcalase and protamex (1:3) method

	pH	Temperature (°C)	Total enzyme concentration (%)	Extraction rate (%)
Level 1	7.0	50	3	
Level 2	7.5	55	4	
Level 3	8.0	60	5	
Experiment 1	7.0	50	3	34.5
Experiment 2	7.0	55	4	37.5
Experiment 3	7.0	60	5	39.3
Experiment 4	7.5	50	4	40.9
Experiment 5	7.5	55	5	41.4
Experiment 6	7.5	60	3	43.0
Experiment 7	8.0	50	5	42.0
Experiment 8	8.0	55	3	44.6
Experiment 9	8.0	60	4	47.8
k_1	37.1	39.1	40.7	
k_2	41.7	41.1	42.0	
k_3	44.8	43.3	40.9	
R	7.7	4.2	1.3	

Nine experiments were designed to cover three factors at three different levels.

also lower for the enzyme methods. Therefore, enzyme methods, in particular a combined enzyme method, should be further explored for possible industrial applications.

3.3. Analysis and characterisation of tea protein

The SDS-PAGE method has been applied to analyse the compositions of the extracted tea protein and five molecule markers were used to mark the molecular weight band (column 1, Fig. 7). Column 3 shows the molecule bands from the tea protein, where seven different protein molecules, with molecular weight of approximately 17.6, 19.7, 25.6, 35.1, 40.5, 46.8 and 72.1 kDa, are clearly identifiable. As a comparison, the protein extracted from fresh tea leaves using a modified acetone method (data not shown) was also examined (column 2). We observe very similar molecular weight distribution for the protein extracted from both sources, except the one at the higher end of molecular weight (129.4 kDa), which appears on the fresh tea leaf but not on the processed one. This may be because this protein is water soluble and has been washed off during the initial hot water soaking process or that this protein has been fully denatured during thermal processing of tea leaves and has become non-extractable.

The amino acid composition of tea protein has also been quantified and the results are shown in Table 6, where the amino acids composition of soy protein is also presented for comparison. We can see that the glutamic acid is the most abundant amino acid in tea protein, followed by aspartic acid, leucine, lysine, alanine and valine. This is more or less in the same order as that of soy protein. The least abundant amino acid in tea protein is cysteine, which only accounts for 0.8%.

Further investigation on the physico-chemical properties and functionalities of tea protein is still underway. Initial results showed that the tea protein had functionalities comparable to those of soy protein, with the potential of being applied as a foaming agent, an emulsifier, a gelling agent and a texture modifier in food applications. Findings on tea protein functionalities will be reported separately.

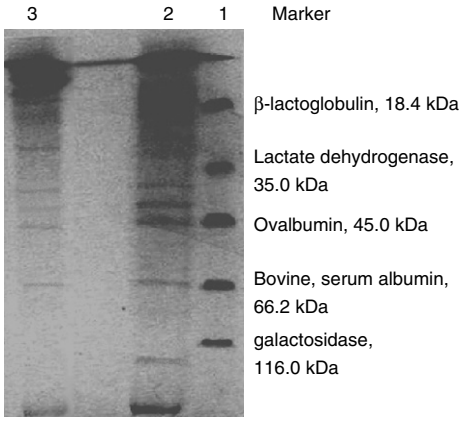

Fig. 7. SDS-PAGE analysis of tea proteins. Column 1 is the molecular markers, column 2 is the protein extracted from fresh tea leaves and column 3 is the protein extracted from processed tea leave pulps.

Table 6
Amino acids composition in tea protein and soy protein (g/100 g protein)

Amino acid	Tea protein	Soy protein
Aspartic acid	8.01	9.9
Threonine	3.83	3.0
Serine	3.94	4.2
Glutamic acid	10.12	17.0
Glycine	4.59	2.4
Cysteine	0.80	4.5
Valine	4.63	1.1
Methionine	1.12	1.1
Isoleucine	3.98	4.1
Tyrosine	3.05	3.2
Phenylalanine	4.46	5.2
Lysine	5.62	5.2
Histidine	1.96	2.3
Arginine	4.83	6.6
Proline	3.62	3.8
Leucine	7.78	6.8
Alanine	4.97	3.4

4. Conclusions

This work demonstrated that both alkaline and enzyme methods were feasible and effective in extracting protein from tea leave pulps. With the help of orthogonal analysis, the optimum extraction conditions have been identified for these extraction methods. It was found that for the alkaline extraction method, an extraction rate of 56.4% could be achieved at 0.1 M NaOH, a volume–weight ratio of 40:1, an extraction time of 5 h and at a temperature of 40 °C. Four different enzymes (neutrase, alcalase, protamex and flavourzyme) were examined for their capability in extracting tea protein. Of the four enzymes, alcalase and protamex were found to be most effective, while the other two gave relatively poorer performance. The combination of alcalase and protamex showed a much increased protein yield. The most significant synergetic effect of the two enzymes was observed at a ratio of 1:3 between alcalase and protamex. At a total enzyme concentration of 4%, a volume–weight ratio of 35:1, an extraction time 4 h, pH 8.0 and temperature 60 °C, an extraction rate of 47.8% was obtained for the mixed enzyme method. Although the enzyme method gave slightly lower protein yield than that of the alkali method, its mild extraction condition and low environment impact make it preferable for tea protein extraction. SDS-PAGE analysis showed the existence of at least seven different types of molecules in tea protein. The composition of amino acid of tea protein has also been analysed and was found to be comparable to that of soy protein.

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