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Antioxidative Activity of Tobacco Leaf Protein Hydrolysates

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Summary

Discarded tobacco leaf protein hydrolysate (DTLPH) was prepared by enzymatic hydrolysis using papain and then separated using ultrafiltration (UF) membranes with molecular mass cut-off (MMCO) of 10, 5, 3 and 1 kDa. Four permeate fractions including 10-K, 5-K, 3-K and 1-K (the permeate fractions from 10, 5, 3 and 1 kDa) hydrolysate fractions were obtained. The 5-K hydrolysate fraction had high oxidation inhibilitory ratio (42.62 %), which was about twofold higher than the original hydrolysate and as high as that of vitamin E (α-tocopherol). The fractionated hydrolysates were superior to the original hydrolysate in the antioxidative activity tested. Moreover, these separated hydrolysates showed the enhanced functional property. The amino acid composition of 5-K hydrolysate was analyzed and the results show that the high antioxidative activity of 5-K hydrolysate was derived from high content of histidine, methionine, cystine and tryptophan.

Key words: discarded tobacco leaf protein, enzymatic hydrolysate, ultrafiltration membrane, antioxidative activity, amino acid composition

Introduction

Over 2 million tonnes of tobacco are harvested in China annually, and approximately 75 % of the total resources are used for producing cigarettes. Furthermore, more than 20 % of the total resources are discarded as processing waste or by-product, which pollutes the environment and is also a waste of reusable material (*1*). In fact, the discarded tobacco leaves are very valuable because their proteins, solanesol, nicotine, amino acids, organic acids and sugar may be widely utilized. One of the approaches for effective utilization of proteins from the discarded tobacco leaf is realized by enzymatic hydrolysis, which is widely applied to improve the functional and nutritional properties of proteins.

For the efficient utilization of proteins from the discarded tobacco leaves, enzymatic hydrolysis using the crude proteinase extracted from the papaya latex should be a good approach (*2*). The molecular mass of the hydrolyzed protein was one of the most important factors influencing the functional properties of the resultant protein hydrolysates (*3*). Therefore, limited enzymatic hydrolysis has been carried out to obtain the hydrolysate with a suitable molecular mass. However, it was impossible to utilize all proteins presented in the tobacco leaf by limited enzymatic hydrolysis because of an insufficient incubation time. In order to produce a hydrolysate or peptide fraction with both a desired molecular mass and a functional property, a useful method was ultrafiltration (UF) membrane system. The major advantage of this system was that the molecular mass distribution of the desired hydrolysates could be controlled by adoption of an appropriate UF membrane (*4,5*).

Lipid peroxidation is an important factor resulting in the quality deterioration of foods during storage. Lipids and lipid-soluble substances that may be susceptible to oxidation are important components of almost all foods. The problem of oxidation is one aspect of food preservation, especially when the oxidative products bring about an unpleasant colour and taste. In food production, it is very important to inhibit or avoid the lipid

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peroxidation in food during processing and storage (*6*). Recently, investigations of antioxidants on resolving lipid peroxidation have been reported. Hydrolyzed proteins from many animal and plant sources, individual peptides and amino acids have been found to possess antioxidant activity. A great number of yeast and soybean protein hydrolysates were used to inhibit the oxidation of corn oil (*7,8*). Some amino acids were reported as having strong antioxidant activity in linoleic acid and methyl linoleate model systems (*9*). It was found in a previous study (*10*) that the content of amino acids in tobacco leaf protein was abundant, so DTLPH was probably a potential antioxidant.

In the present study, the fractions of the hydrolysate of the discarded tobacco leaf protein using UF membranes were described. The characterization of the separated fractions with antioxidative activity was carried out. Molecular mass distribution of the desired hydrolysates was studied by HPLC system, and amino acid composition of the desired hydrolysates was analyzed.

Materials and Methods

Materials

The discarded tobacco leaf protein was extracted from discarded tobacco leaf in two stages. The first stage: the tobacco leaf/water ratio of 1:17, 60 °C, pH=8.0; ground twice. The second stage: extraction was carried out under optimal conditions: 60 °C, pH=8.0, 60 min; extracted three times with agitation; the conditions of depositing protein using an acid were: pH=3.0, 4 °C and 8 h. The extraction rate reached 86.62 % under the optimal conditions (data not shown). The discarded tobacco leaf protein mixture was composed of 60.36 % of water and 39.64 % of solids (28.12 % of protein and 11.52 % of cellulose). Papain (from papaya latex, 4.0×10^5 U/g) was purchased from Guangzhou Enzyme Co. Ltd., PR China. Thiobarbituric acid (TBA) and linoleic acid for the measurement of antioxidative activity were purchased from Sigma. The standard used contained the following protein fractions: cytochrome C (M_r=12 400 Da), aprotinin (M_r=6500 Da), bacitracin (M_r=1400 Da) and methionine (M_r=556 Da). All other chemicals used were of analytical grade.

Preparation of DTLPH

According to an enzyme/substrate ratio of 1:100 (by mass), 5 % of substrate (dry mass basis of the protein attached to discarded tobacco leaf) in 10 L of phosphate buffer (pH=6.0) and papain were mixed. The mixture was incubated for 6 h at 50 °C with stirring and then heated in a boiling water bath for 15 min to inactivate the enzyme at 100 °C. DTLPH was then rapidly cooled to room temperature in the ice bath. One of the resulting solutions was set aside for measurement of antioxidative activity and the other was first passed through the 10-kDa molecular mass cut-off (10-K MMCO) membrane. The ultrafiltration system (Minitan™ System) and membranes for the fractionations of each hydrolysate were purchased from Millipore. A portion of the solution was removed immediately and the filtrate was then pumped through 5-, 3-, and 1-K MMCO membranes in order to decrease pore size. The respective permeate passed through each membrane and the 10-K DTLPH (permeate from 10 kDa MMCO), 5-K DTLPH (permeate from 5 kDa MMCO), 3-K DTLPH (permeate from 3 kDa MMCO), and 1-K DTLPH (permeate from 1 kDa MMCO) were freeze--dried.

Molecular mass distribution profile

Molecular mass distributions of the hydrolysates were determined by the gel permeation chromatography (GPC) using an HPLC system (Waters 1525 Binary HPLC Pump, Waters 2487 Dual λ Absorbance Detector). GPC column (Protein-Pak 60) (7.8 mm i.d.×30 cm, 60 Å) was connected in series. The hydrolysates were separated at room temperature under isocratic conditions (0.05 mol/L Tris--HCl buffer, pH=7.2) and monitored at 220 nm and flow rate of 0.7 mL/min. Normal curve equation of molecular mass was as follows:

$$M_r(\mathrm{lg})=5.434-0.159\cdot t \quad (R^2=0.999) \qquad /1/$$

where M_r is molecular mass and t is retention time.

Antioxidative activity

Antioxidative activity was determined by the thiobarbituric acid (TBA) assay with an aliquot of the oxidized linoleic acid solution. The oxidation of linoleic acid was conducted as described by Osawa and Namiki (*11*) with slight modifications. A volume of 0.2 mL of the sample of linoleic acid was added to a 30-mL test tube, and diluted with 10 mL of absolute alcohol and 10 mL of 50 mmol/L phosphate buffer (pH=7.0). Each sample (2 mg) was added to the mixed solution and the total volume was adjusted to 25 mL with distilled water. The mixture was incubated in the dark at 40 °C for 6 days. TBA assay was carried out as described by Ohkawa *et al*. (*12*) and Stocks and Domandy (*13*) on the 6th day with some modifications. The solution containing 0.8 mL of water, 0.2 mL of 8.1 % SDS and 1.5 mL of 20 % acetic acid solution was adjusted to pH=3.5 with 10 M NaOH and 1.5 mL of 0.8 % TBA. A total of 50 mL of the oxidized linoleic acid solution was added to the mixture and incubated at 5 °C for 1 h, and then heated at 100 °C for 1 h. The concentration of red pigment was estimated by absorbance at 535 nm. The result was expressed as the oxidation inhibitory ratio for linoleic acid and compared to α-tocopherol.

Amino acid composition analysis

Amino acids were analyzed by HPLC system using the method of Lindroth and Mopper, according to Ingalls *et al.* (*14*). A Pico-Tag™ column (3.9 mm i.d.×15 cm) equipped with a guard column was eluted at a flow rate of 1.0 mL/min and 38 °C. A binary gradient of 0.05 M sodium acetate (pH=5.7), 5 % THF (eluant A) and methanol (eluant B) was used, increasing from 22 to 50 % B in 20 min, then to 100 % B in 10 min. OPA-derivatized amino acids were detected at 254 nm and identified by comparison with retention time of authentic standards, amino acid identifications were verified in a few samples by GC-MS. Aspartic acid and glutamic acid measurements included the hydrolysis products of asparagine and glutamine.

Statistical analysis

Microcal Origin ver. 6.1 software (Microcal software, Northampton, USA) was used for statistical analysis of means and standard deviations. Duncan's multiple range test ($p<0.05$) using SAS procedures (Release 6.08, SAS Institute Inc., Cary, NC, USA) was used to detect significant difference in different mean values.

Results and Discussion

Molecular mass distribution of DTLPH

Recently, HPLC system has been used widely in the separation of the proteins and polypeptides. This way of purification also had a great potential value in the early stages of peptide purification (*15*). In this study, HPLC system was used to separate peptides of DTLPH. The analysis of this hydrolysate was performed by the HPLC system (Fig. 1, Table 1). The hydrolysate had seven fractions and the molecular mass distribution was less than 10 000 Da. In these seven fractions, the fraction of 3436 Da was the major peak and the peak area was 46.29 % of the total fractions (Fig. 1, Table 1).

Table 1. Molecular mass distribution of DTLPH

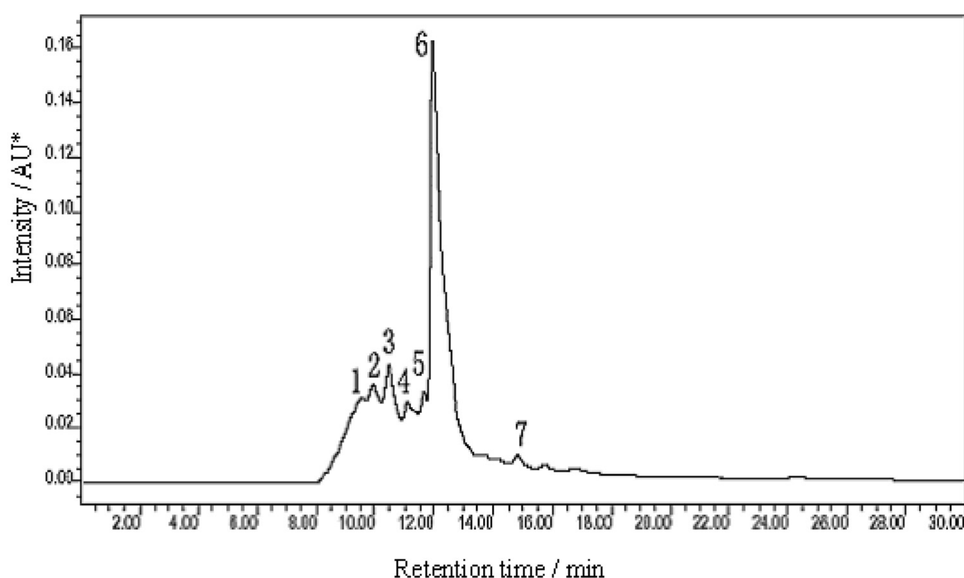
Fraction	Retention time/min	Peak area/%	M_r/Da
1	9.230	15.20	9256
2	9.907	10.80	7224
3	10.446	14.74	5930
4	11.078	7.29	4705
5	11.649	4.43	3818
6	11.937	46.29	3436
7	14.810	1.25	1200

Antioxidative activity of DTLPH

Only a few reports on the antioxidative effect of amino acids have been published. Different composition of amino acids showed different degree of antioxidative activity (*16*). However, all amino acids showed antioxidative activity in some systems, which probably reflected the antioxidative nature of the NH_3R group (*17*). The use of a protein or a hydrolysate for the improvement of the antioxidative activity in functional material fields might be more practical than the use of amino acids, because proteins and enzymatic hydrolysates had many other functional properties. Soybean protein hydrolysates obviously had antioxidative effects (*18–20*).

In the present study, DTLPH was separated by using four kinds of UF membranes (10-, 5-, 3- and 1-kDa MMCO membranes) according to the molecular size, and four kinds of permeates (10-, 5-, 3- and 1-K hydrolysates) were obtained. The oxidation inhibitory ratio of the four hydrolysate fractions derived from DTLPH was compared to the original hydrolysate and α-tocopherol, which had been widely used as a natural antioxidative agent. Obviously, the 5-K hydrolysate possessed the most effective antioxidative activity and showed about twofold higher activity than the original hydrolysate (Fig. 2). The activity was nearly as high as that of α-tocopherol.

DTLPH produced after the hydrolysis for 6 h with papain showed the highest activity, which decreased with reaction time (data not shown). The strongest activity (42.62 %) of oxidation inhibitory ratio was obtained in the fraction of 5-K hydrolysate. These results indicate that the antioxidative activity of proteins or peptides depended on their molecular mass.



*AU=arbitrary units

Fig. 1. Molecular mass distribution profile of DTLPH. It was fractionated depending on the molecular mass of peptides. The resultant hydrolysate was separated on high performance gel permeation with the GPC column (GPC 7.8 mm i.d.×30 cm), connected in series. The standard used contained the following protein fractions: cytochrome C (M_r=12 400 Da), aprotinin (M_r=6500 Da), bacitracin (M_r=1400 Da) and methionine (M_r=556 Da)

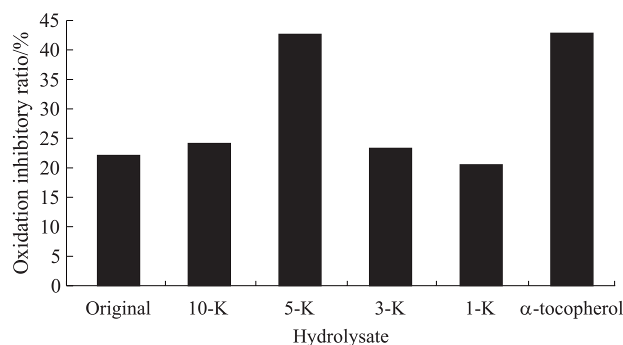


Fig. 2. Oxidation inhibitory ratio of DTLPH was determined by TBA method in linoleic acid oxidation system. Oxidation inhibitory ratio was determined on the 6th day. Vitamin E: α-tocopherol; original: original hydrolysate; 10-K: 10-K DTLPH (permeate from 10 kDa MMCO); 5-K: 5-K DTLPH (permeate from 5 kDa MMCO); 3-K: 3-K DTLPH (permeate from 3 kDa MMCO); 1-K: 1-K DTLPH (permeate from 1 kDa MMCO). The values are the means of at least two determinations

Molecular mass profile of 5-K hydrolysate

The pattern of the molecular mass distribution of 5-K hydrolysate was similar to that of the original hydrolysate, although the relative proportions of the peaks varied according to the MMCO size of the membrane used. The 5-K hydrolysate had a molecular mass distribution of less than 10 000 Da, and the major peak of 5-K hydrolysate was located at 3442 Da, which was the highest proportion (62.36 %). It showed that high antioxidative ability of 5-K hydrolysate was associated with the fraction of 3442 Da.

Analysis of amino acid composition

It was reported that tobacco leaf protein contained abundant amino acids, such as aspartic acid, glutamic acid, glycine, alanine, threonine and serine (*10*). In this paper, the peptide fractions isolated by UF membranes were analyzed on a Pico-Tag^{TM} column (3.9 mm i.d.×15 cm) after HCl and NaOH hydrolyses, converting their amino acids into their PTC (phenylthiocarbamyl) derivatives (*21*).

Table 2 shows the amino acid composition of both original and 5-K hydrolysate. Amino acid abundance of original hydrolysate followed the order: glutamic acid, aspartic acid, arginine, leucine, proline, glycine,…, histidine, methionine, cystine. Amino acid abundance of 5-K hydrolysate followed the order: glutamic acid, aspartic acid, tryptophan, leucine, proline, arginine,…, alanine, methionine, cystine. The content of glutamic acid was the highest and the content of cystine was the lowest in all the amino acids of the original and 5-K hydrolysates. From Table 2 it can be seen that 5-K hydrolysate (3442 Da) had higher content of in histidine, methionine, cystine and tryptophan than the original hydrolysate.

The antioxidative effect of amino acids has been reported in recent studies, for example, tryptophan and histidine showed high activity, whereas glycine and alanine showed only weak activity, and methionine and cysteine had antioxidative effect in soybean oil (*22–25*). Uchida and Kawakishi (*26*) reported the antioxidative activity of histidine-containing peptide, and showed that this peptide had a higher activity because the imidazole ring of histidine probably contributed to the chelating ability and the lipid radical-trapping ability. In this study, antioxidative effect of 5-K hydrolysate was higher than that of the original hydrolysate, as high as that of α-tocopherol (Fig. 2), primarily because of the higher content of bioactive amino acids (*22–25*), such as histidine, methionine, cystine and tryptophan (Table 2). Consequently, DTLPH could be widely used in food industry as a potential natural antioxidant.

Table 2. The amino acid composition of DTLPH (mg/g protein)

Amino acid	*w*(original hydrolysate) (mg/g)	*w*(5-K hydrolysate) (mg/g)
Aspartic acid + aspartine	60.58	49.52
Glutamic acid + glutamine	98.56	75.59
Serine	45.30	29.63
Glycine	45.99	29.15
Histidine	14.93	24.85
Arginine	49.57	39.56
Threonine	41.39	38.24
Alanine	43.64	8.10
Proline	47.50	40.06
Tyrosine	37.62	28.13
Valine	39.00	37.66
Methionine	3.73	5.79
Cystine	1.02	1.86
Ileucine	30.91	25.45
Leucine	48.12	40.90
Tryptophan	26.19	47.94
Phenylalanine	36.09	22.71
Lysine	32.92	25.11

Conclusions

The discarded tobacco leaf resources could be utilized in enzymatic hydrolysis, which has been widely applied to improve the functional and nutritional properties of proteins. Discarded tobacco leaf protein hydrolysate could be separated by using UF membranes instead of chromatographic processing. The hydrolysates separated using UF membranes showed some advantages including mass production of the desirable fractions and enhancement of some functionalities in comparison with the original hydrolysate, together with simplification of the separation process and reduction in the cost of production compared to chromatographic processing. The 5-K DTLPH showed high antioxidative activity and the fractionated hydrolysates were superior to the original non-separated hydrolysate. Moreover, antioxidative activity was associated with molecular mass. The analysis of amino acid composition implied some potential bioactivities of peptides. In this case, 5-K DTLPH exhibited high antioxidative activity due to the high content of histidine, methionine, cystine and tryptophan. As a potential natural antioxidant, it is expected to be used in the food processing.

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