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ScienceDirect
Food Chemistry 109 (2008) 144–148
Food Chemistry
www.elsevier.com/locate/foodchem

Rapid Communication

Antioxidant activity and water-holding capacity of canola protein hydrolysates

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Received 9 September 2007; received in revised form 15 November 2007; accepted 16 December 2007

Abstract

Canola protein hydrolysates were prepared using commercial enzymes, namely Alcalase, an *endo*-peptidase and Flavourzyme with both *endo*- and *exo*-peptidase activities. The hydrolysates so prepared were effective as antioxidants in model systems, mainly by scavenging of free radicals and acting as reducing agents. This effect was concentration-dependent and also influenced by the type of enzyme employed in the process. The hydrolysate prepared using flavourzyme showed the highest antioxidant activity among all samples, whereas the hydrolysates prepared by combination of Alcalase and Flavourzyme did not differ significantly ($P > 0.05$) in antioxidant effectiveness from that produced by Alcalase alone. The hydrolysates were also found to be effective in enhancing water-holding capacity and cooking yield in a meat model system. Their capability in improving the cooking yield of meat was in the order of Flavourzyme hydrolysates > combination hydrolysates > Alcalase hydrolysates. These results suggest that canola protein hydrolysates can be useful in terms of their functionality and as functional food ingredients and that their composition determines their functional properties and thus their potential application in the food and feed industries.

Keywords: Canola protein hydrolysate; Alcalase; Flavourzyme; Antioxidant activity; Water-holding capacity

1. Introduction

Rapeseed is placed as one of the top five oilseed crops in the world and thus is of great importance to the global agricultural industry. Canola, the Canadian variety of rapeseed, is in its own right a major resource for North American agriculture and it is low in both glucosinolate and erucic acid, thus also known as "double zero" variety. Its seeds and oils are exported to many parts of the world, including the United States, Mexico, Japan, China and Europe. The global demand for canola is mainly for its edible oil. However, once the oil is removed from the seed, a protein-rich meal is left behind. Considering the large amount of byproduct produced by the extraction process, it would be greatly beneficial to the agricultural industry to develop techniques to use this protein source for developing value-added products. In order to do so, efforts in practice may be hampered by many factors, such as the presence of phytates and glucosinolates, as well as the fact that the commercial extraction process denatures the protein and, as a result, decreases its solubility (Vioque, Sánchez-Vioque, Clemente, Pedroche, & Millan, 2000). These factors make it almost impractical to use canola/rapeseed protein in any meaningful way for human food and hence the current use of rapeseed meals is generally restricted to animal feed and fertilizer.

Many investigations have focussed on utilization of canola protein for production of valuable food supplement. Some studies have focussed on removing the undesirable components from the meal, a process that has proven to be complicated (Diosday, Rubin, Philips, &

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doi:10.1016/j.foodchem.2007.12.039

Naczk, 1985; Dunford & Temelli, 1996; Shahidi, Gabon, Rubin, & Naczk, 1990; Shahidi, Naczk M., Rubin, & Diosady, 1988; Tzeng, Diosady, & Rubin, 1990). Others have focussed on the solubility issues (Mahjan, Dua, & Bhardwaj, 2002; Radwan & Lu, 1976). Thus, a method was needed to modify the physical and chemical characteristics of the protein meal without altering its overall amino acid composition. Further investigations demonstrated that simple hydrolysis, either by chemical or enzymatic means, was a potential solution to the solubility problem (Adler-Nissen,1986). Hydrolysis of a protein is a simple and inexpensive method to convert a protein into free amino acids and short chain polypeptides. Such products are far more soluble in water than the original protein yet their amino acid composition remains essentially unchanged.

A vast number of studies have been conducted on the preparation of hydrolysates from different protein sources in an effort to better understand their properties. These include capelin protein hydrolysates (Amarowicz & Shahidi, 1997; Shahidi & Amarowicz, 1996; Shahidi, Han, & Synowiechi, 1995), seal protein hydrolysates (Shahidi, Synowiecki, & Balejko, 1994), casein protein hydrolysates (Mahmoud, Malone, & Cordle, 1992), whey protein hydrolysates (Turgeon, Gauthier, & Paquin, 1992) and sunflower seed protein hydrolysates (Conde, Escobar, Jiménez, Rodríguez, & Patino, 2005). In general, the results tend to show that hydrolysis increases solubility of proteins to varying extents, depending on protein composition and the degree of hydrolysis. Moreover, protein hydrolysates produced may possess some physicochemical characteristics and bioactivities not found in the original proteins, such as antioxidant activity and water-holding capacity. For instance, Shahidi et al. (1995) reported that capelin protein, at a level of 0.5–3.0%, inhibited the formation of thiobarbituric acid-reactive substances (TBARS) by 17.7–60.4% in a cooked pork model system. A similar effect was documented for potato protein hydrolysates (Wang & Xiong, 2005). Protein hydrolysates from seal meat were found to improve water-holding capacity in meat products (Shahidi & Synowiecki, 1997). In addition to antioxidant activity and water-holding capacity, angiotensin I converting enzyme (ACE) inhibitory activity was also reported for bovine skin gelatin hydrolysate (Kim, Byun, Park, & Shahidi, 2001).

Hydrolysis has also been proposed for better use of the rapeseed meal and studies have been carried out to evaluate the hydrolysis products. Vioque et al. (1999) and Vioque et al. (2000) have investigated rapeseed protein hydrolysates produced under different conditions. In this study, the antioxidant activity of canola protein hydrolysates prepared by two different proteases, namely, Alcalase, an *endo*-peptidase and Flavourzyme, a mixture of *endo*-peptidase and *exo*-peptidase, was evaluated. The water-holding capacity in a meat model system, as affected by each hydrolysate, was also examined.

2. Materials and methods

2.1. Materials

Seeds of Cyclone canola used in this study were grown in several locations in western Canada and were procured from Limagrain Canada Seeds Inc., Saskatoon, SK. The seeds were bulked, sub-sampled and then stored at −18 °C until used. Alcalase and Flavourzyme were purchased from Novozymes, Bagsvaerd, Denmark. All chemicals used were obtained from Fisher Scientific Ltd. (Ottawa, ON, Canada) or Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). The solvents used were of ACS grade, pesticide grade or HPLC grade and were used without any further purification.

2.2. Preparation of canola protein hydrolysates

Whole canola seeds were ground and defatted with hexane, following a small-scale hexane extraction method described by Tzeng et al. (1990). The defatted canola meal samples were vacuum-packed and stored at −20 °C prior to hydrolysis. The crude protein content in the meal was determined by Kjeldahl analysis according to the AOAC (1990) method in order to calculate the amount of sample required for the hydrolysis process, based on enzyme/protein ratio. The meal samples were divided into three groups (each containing 29 g of canola meal and 200 ml of water) and hydrolyzed under pre-selected conditions according to Vioque et al. (1999) with some modifications. Sample 1 was hydrolyzed at 50 °C and pH 8 for 1 h using Alcalase (1.05 ml) and sample 2 at 50 °C and pH 7 for 2 h using Flavourzyme (0.40 ml). Finally, sample 3 was produced, using a combination of the two enzymes, i.e. hydrolyzing first with Alcalase for 1 h, followed by Flavourzyme for an additional 2 h (Table 1). Conditions were constantly monitored and maintained throughout the process. The pH value, which changes as a result of hydrolysis, was kept constant by the addition of a known amount of 4N NaOH. Upon completion of the hydrolysis, the enzymes were deactivated by dropping the pH to 5. The reaction mixtures were then filtered and the hydrolysates collected. The protein hydrolysates obtained were freeze-dried and stored at −20 °C for subsequent analysis.

2.3. DPPH radical-scavenging assay

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging activity of enzymatically prepared canola protein hydrolysates was determined, following the procedure described by Shahidi, Liyana-Pathirana and Wall (2006) with minor modifications. Freeze-dried hydrolysate samples were dissolved in 95% ethanol at a series of concentrations (1.25, 2.5, 5 and 10 mg/ml). An aliquot (0.1 ml) of the sample solution was mixed with 1.9 ml of ethanolic DPPH$^{\cdot}$ solution (50 μM) and the mixtures were allowed to stand at room temperature for 30 min. The absorbance was then

Table 1
Hydrolysis conditions used for preparation of canola protein hydrolysates

Sample	Enzyme	pH	Temperature (°C)	Time (min)	E/P[a]
1	AL[b]	8	50	60	0.3 AU/g
2	FL[c]	7	50	120	50 LAPU/g
3	Combined enzymes	8 7	50 50	60 (AL) 120 (FL)	0.3 AU/g 50 LAPU/g

[a] E/P: enzyme/protein.
[b] AL: Alcalase.
[c] FL: Flavourzyme.

read at 517 nm using a spectrophotometer and the scavenging of DPPH· by protein hydrolysates was calculated as follows:

$$\text{scavenging} = 100 \times \left[\text{Abs}_{\text{control}} - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})/\text{Abs}_{\text{control}}\right]$$

where $\text{Abs}_{\text{control}}$ is the absorbance of DPPH· without any protein hydrolysate, while $\text{Abs}_{\text{blank}}$ represents absorbance of protein hydrolysates without DPPH·, since proteins absorb at this wavelength.

2.4. Determination of reducing power

Reducing power of canola protein hydrolysates was measured according to Duh, Yen, Yen and Chang (2001) with some modifications. Briefly, canola protein hydrolysates samples were dissolved in a 0.2 M phosphate buffer (pH 6.6) at concentrations of 1.25, 2.5, 5 and 10 mg/ml. An aliquot (2.5 ml) of sample solution was then added to 2.5 ml of a 10 mg/ml potassium ferricyanide solution and incubated at 50 °C for 20 min. To the mixtures, after incubation, deionized water (2.5 ml) and a ferric chloride solution (1.0 mg/ml, 0.5 ml) were added. The absorbance was then recorded immediately at 700 nm. The trichloroacetic acid step was omitted since it would precipitate out the protein whose antioxidant activity is being assessed. A control, devoid of any hydrolysates and a blank, containing only hydrolysate samples, were used because proteins also absorb at the same wavelength. Increased absorbance of the reaction mixture indicated increased reducing power.

2.5. Determination of water-holding capacity

Water-holding capacity of canola protein hydrolysates in a meat model system was determined according to Shahidi and Synowiecki (1997). To a mixture containing 8.5 g of ground pork and 1.5 g of distilled water, canola protein hydrolysates were added at 0.5 and 1% (w/w) levels and mixed thoroughly. A control without any protein hydrolysate was also prepared. The mixture was allowed to stand in a cold room for 1 h and subsequently cooked at 95 °C in a water bath for 1 h followed by cooling under a stream of cold tap water. The drip water was removed with a filter paper and the weight of the meat was recorded. The drip volume was obtained by calculating the weight loss after cooking. Water-holding capacity for different protein hydrolysates was expressed as decrease of drip volume against a control.

3. Results and discussion

The defatted canola meal had a protein content of 34.5% by weight. Based on the amount of base consumed to keep the pH constant during the course of hydrolysis, the sample hydrolyzed by Alcalase and that hydrolyzed by a combination of Alcalase and Flavourzyme had similar degrees of hydrolysis (20.6% and 18.9%, respectively), while the hydrolysate prepared with Flavourzyme alone had the lowest degree of hydrolysis (6.33%). This was unexpected as the combination of Alcalase and Flavourzyme was thought to produce a more extensively hydrolyzed product than using one enzyme alone, as observed by Vioque et al. (1999), who indicated that the rapeseed was hydrolyzed to a greater extent using both enzymes than either of them individually. In this study, Alcalase and Flavourzyme combination produced a DH similar to that of Alcalase alone. It is possible that Flavourzyme in the combination was not very active and it either did not hydrolyze or hydrolyzed canola meal only to a limited extent.

The results of both antioxidant tests employed, namely, DPPH radical-scavenging capacity and reducing power, showed that all canola protein hydrolysates examined possessed antioxidant activity which was dependent on the hydrolysis treatment employed. Table 2 shows the DPPH radical-scavenging capacity of canola protein hydrolysates prepared by different enzymes. All three hydrolysate samples exhibited a concentration dependent scavenging activity against DPPH radicals. Their scavenging capacity increased with increasing concentration. The hydrolysates prepared using Flavourzyme had the highest scavenging activity at all concentrations, while similar values were obtained for hydrolysates prepared using Alcalase or the combination of the two enzymes. Statistical analysis

Table 2
The scavenging capacity of DPPH radicals (%) by various concentrations of different Canola hydrolysates[A]

Concentration (mg/ml)	1.25	2.5	5	10
Hydrolysate by AL[B]	16.8 ± 0.68^{b}	28.9 ± 0.88^{b}	48.7 ± 2.10^{ab}	70.2 ± 0.95^{b}
Hydrolysate by FL[C]	22.0 ± 1.90^{a}	34.8 ± 1.30^{a}	52.1 ± 1.50^{a}	73.2 ± 1.30^{a}
Hydrolysate by AL + FL	18.9 ± 0.99^{ab}	30.1 ± 0.92^{b}	48.1 ± 0.98^{b}	69.1 ± 1.20^{b}

[A] Results reported are means of triplicate samples ± standard deviation. Values in the same column with different superscripts are significant different at $P < 0.05$.
[B] AL: Alcalase.
[C] FL: Flavourzyme.

revealed that the difference between the hydrolysate prepared with the aid of Flavourzyme and the other samples was significant ($P < 0.05$), but those for the use of Alcalase and the combination enzyme system did not differ significantly ($P > 0.05$) in their DPPH·-scavenging ability.

The differences in DPPH·-scavenging capacity, as well as the similarities, are rather revealing. First, the difference between the hydrolysates using Flavourzyme and those using Alcalase is a good indicator that hydrolyzing of proteins/peptides by different enzymes will alter the antioxidant potential of products. Studies have shown that the antioxidant capacity of a peptide depends on its composition. For instance, tripeptides with tryptophan or tyrosine at their C-terminus exhibit very strong radical-scavenging activity (Saito et al. 2003). Different combinations of amino acids in tripeptide chains also exhibited different antioxidant activities (Saito et al., 2003). Since radical-scavenging is likely due the hydrogen donor activity of the hydroxyl groups of aromatic amino acids, the presence or absence of such amino acids in the peptide would affect its scavenging capacity. Therefore, hydrolysis of the polypeptide in various portions may result in varied radical-scavenging activity of the hydrolysates. It is known that Alcalase and Flavourzyme hydrolyze proteins in different manners, Alcalase acts as an *endo*-peptidase, thus cleaving peptide bonds at the interior of the polypeptide chain (Adler-Nissen, 1986), Flavourzyme has both *endo*-peptidase and *exo*-petidase activities. Therefore, Alcalase produces mainly small- and medium-size oligopeptides/polypeptides while Flavourzyme produces more low-molecular-weight components, e.g. small to medium size peptides, as well as amino acids (Hamada, 2000). The composition of the hydrolysates prepared using Flavourzyme and Alcalase determines their functional characteristics and thus may account for their varied antioxidant activity. The fact that protein hydrolysates, in this work prepared by Alcalase and by combination of Alcalase and Flavourzyme, had similar DPPH·-scavenging abilities further supports the postulate that, in the combination, Flavourzyme probably did not contribute or contributed little to the hydrolysis of the canola protein meal.

The results of the reducing power of the hydrolysates correlated well with those of the DPPH·-scavenging assay, as shown in Table 3. Reducing power of canola protein hydrolysates appeared to be concentration-dependent and increased with increasing concentration. Reducing power is a measure of global antioxidant capacity as antioxidants show reducing power in redox reactions. Like the radical-scavenging capacity, the reducing power of canola protein hydrolysate varied, depending on the enzymes used for hydrolysis. Flavourzyme hydrolysate displayed the highest reducing power among all samples tested. The hydrolysates from the action of Alcalase and combined enzymes did not differ significantly ($P > 0.05$) in their reducing powers. A strong correlation existed between the DPPH·-scavenging capacity and reducing power of the hydrolysates, thus suggesting that the antioxidant activity of enzymatically prepared canola protein hydrolysates is composition-dependent and can be altered by changing the type of enzyme used in the hydrolysis process. Although protein hydrolysates were the main focus of study in this work, other components in canola meal, such as some phenolics, might be co-extracted during the preparation of hydrolysates and could also contribute to the antioxidant activity of the crude extracts. Further research on purification of the protein hydrolysates from the crude extracts needs to be conducted in order to eliminate/minimize interference from other potentially antioxidative constituents.

Table 3
Reducing power of the hydrolysate samples at different concentrations measured as absorbance ($\times 10^3$) at 700 nm[A]

Concentration (mg/ml)	1.25	2.5	5	10
Hydrolysate by AL[B]	2.06 ± 4.97	20.2 ± 3.72^{b}	61.4 ± 37.9	73.4 ± 5.70^{ab}
Hydrolysate by FL[C]	20.6 ± 17.0	39.1 ± 7.83^{a}	67.9 ± 6.62	91.7 ± 10.0^{a}
Hydrolysate by AL + FL	6.76 ± 6.63	22.8 ± 2.46^{b}	43.5 ± 19.1	64.3 ± 12.3^{b}

[A] Results reported are means of triplicate samples ± standard deviation. Values in the same column with different superscripts are significant different at $P < 0.05$.
[B] AL: Alcalase.
[C] FL: Flavourzyme.

With respect to water-holding capacity, all canola protein hydrolysates enhanced the water-holding capacity of the meat and thus improved cooking yield (Table 4). This effect was also concentration-dependent and influenced by the enzymes employed during the hydrolysis process. Among the three hydrolysates, Flavourzyme hydrolyzate was most effective in decreasing the drip volume, followed by hydrolysates prepared by combination of Alcalase and Flavourzyme and least effective was the one prepared by Alcalase alone.

Owing to the hydrolysis pattern of Flavourzyme and its tendency to produce low-molecular-weight peptide fragments, it appears that lower-molecular-weight peptides are more effective in holding water than are larger-size peptides. This is possibly because smaller fragments of peptides would be more hydrophilic. However, composition of the

Table 4
Decrease of drip volume (%) in a meat system by various concentrations of canola protein hydrolysates[A]

Concentration (%, w/w)	0.5	1.0
Hydrolysate by AL[B]	4.10 ± 0.19^{c}	8.67 ± 0.18^{c}
Hydrolysate by FL[C]	12.5 ± 0.55^{a}	14.2 ± 0.22^{a}
Hydrolysate by AL + FL	8.43 ± 0.09^{b}	10.6 ± 0.56^{b}

[A] Results reported are means of triplicate samples ± standard deviation. Values in the same column with different superscripts are significant different at $P < 0.05$.
[B] AL: Alcalase.
[C] FL: Flavourzyme.

peptides in each of the fractions may also play an important role. If hydrolysis via Flavourzyme preserved more of the hydrophilic amino acid residues, then the hydrolysate produced would also tend to exert a better water-holding capacity. Further research should be conducted to examine the exact amino acid compositions of the hydrolysates so produced and their relationship to water-holding capacity of meat products.

In conclusion, canola protein hydrolysates were found to possess antioxidant activity and improved water-holding capacity of meat products. The composition of enzymatically prepared protein hydrolysates dictates their functional properties and thus their potential application in the food and animal feed industries. Among the hydrolysates examined, those prepared using Flavourzyme were superior in terms of their antioxidant activity and water-holding capacity. Further studies are needed to identify the peptide fractions responsible for the antioxidant potential and water-holding capacity of the products.

Acknowledgement

We are grateful to the Natural Science and Engineering Council (NSERC) of Canada for financial support in the form of a discovery grant to FS.

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