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CHYMOTRYPTIC HYDROLYSIS OF LENTIL MEAL PROTEINS AND CHARACTERISTICS OF THE RESULTING HYDROLYSATES

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Key words: lentil, chymotrypsin, degree of hydrolysis, hydrolysates, SDS-PAGE, SE-HPLC, astringency, bitterness

Lentil meal proteins were treated by chymotrypsin. Hydrolysis was controlled with the pH-stat method. Degree of hydrolysis (DH) was evaluated and after 120 min of the process amounted to 13%. SDS-PAGE and SE-HPLC methods were used to study molecular weight distribution of lentil meal proteins and their chymotryptic hydrolysates of DH 2%, 4%, 8% and 12%. Bands in the range of 21–66 kDa were predominant on the electrophoregram of lentil proteins. Chymotrypsin treatment resulted in releasing the hydrolysis products of both high molecular weight (62,000; 30,500 Da) molecules and small peptides (<6,500 Da). At the first stage of hydrolysis (DH 2.0%) intermediate products are formed, which are then further hydrolysed. Chromatographic separation confirmed the results of SDS-PAGE. Larger polypeptides and unhydrolysed proteins are present in hydrolysate of DH 12% but products of hydrolysis with molecular weight of 0.5–6.5 kDa are predominant. No simple correlation between degree of hydrolysis and intensity of bitterness and astringency sensation was noticed. Bitterness of hydrolysates was not high (2.25-2.35 a.u.).

INTRODUCTION

Enzymatic hydrolysis changes the chemical, physical, biological, and immunological properties of proteins. Protein hydrolysates are digested easier and more rapidly than the intact proteins. It was also noticed that di- and tripeptides can be adsorbed faster into the gastrointestinal tract than free amino acids [Frokjaer, 1994]. For this reason, protein hydrolysates are widely used as nutritional supplements and substitute ingredients of specially designed diets for sportsmen, the elderly and people on a slimming diet [Lahl & Braun, 1994; Mahmoud, 1994]. They are also constituents of special medical diets applied in Crohn's disease, ulcerative colitis, short bowel syndrome, fistulas, pancreatitis *etc*. [Schmidl *et al*., 1994]. Hydrolysates of a high degree of hydrolysis, those containing short polypeptide chains, have lower allergenic potential than large oligopeptides and intact proteins [Mahmoud, 1994; Wróblewska *et al*., 2004]. Furthermore, it was reported that some hydrolysates, *i.e.* obtained from pea and soy proteins, corn gluten, and muscle food industry by-products, exhibit inhibitory activity of angiotensin I converting enzyme (ACE) [Humiski & Aluko, 2007; Lo & Li-Chan, 2005; Kim *et al*., 2004; Karamać *et al*., 2005]. It is possible to generate hydrolysate products with either enhanced or reduced functionality, *e.g.*, solubility, viscosity, emulsification, foaming and gelating properties [Jung *et al*., 2005; Chabanon *et al*., 2007; Guan *et al*., 2007; Kong *et al*., 2007]. The extent of alterations depends on the degree of hydrolysis (DH) achieved, which is affected by enzyme specificity, physical state and chemical characteristics of intact protein, and hydrolysis conditions. Hydrolysates are also believed to possess antioxidant activity [Shahidi & Amarowicz, 1996; Pena-Ramos & Xiong, 2002; Humiski & Aluko, 2007; Wang *et al*., 2007].

Hydrolysis significantly impacts the sensory properties of the resultant hydrolysates. Bitterness is a negative attribute associated with release of low molecular weight peptides containing hydrophobic amino acid residues during proteolysis [Adler-Nissen, 1984; Saha & Hayashi, 2001]. The higher the degree of hydrolysis, the more hydrophobic peptides are released, which evokes an increase in bitterness [Adler-Nissen, 1984; Slattery & Fitzgerald, 1998; Seo *et al*., 2008]. However, while fractionating peptides from soy protein hydrolysates Cho *et al*. [2004] noticed that the peptides with molecular weight of 2–4 kDa showed higher bitterness than the peptide fraction with molecular weight of <1,000 Da. Specific debittering strategies have been developed and bitterness of hydrolysetes can be now effectively decreased or eliminated. Saha & Hayashi [2001] reviewed numerous options in the debittering of food protein hydrolysates.

Lentil protein can be a potential material to obtain protein hydrolysates. Lentil seeds contain more protein than other legume seeds *i.e*. bean or pea. Protein content of lentil seeds ranges from 24.3% to 30.2% for different cultivars [Wang & Daun, 2006]. Lentil protein is valuable and contains all essential amino acids, but the content of tryptophan and sulphur-containing amino acids (methionine and cysteine) is slightly lower than FAO/WHO requirement patterns [Wang & Daun, 2006].

The aim of the present study was to hydrolyse lentil meal proteins using chymotrypsin under controlled conditions,

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to obtain hydrolysates of specific degree of hydrolysis and to characterise molecular weight distribution of hydrolysates and their astringency and bitterness in relation to degree of hydrolysis.

MATERIALS AND METHODS

Materials

Lentil seeds (*Lens culinaris*) cultivar Tina were purchased in Plant Breeding Station "Spójnia" (Śrem, Poland). Seeds were ground using a laboratory mill until particles smaller than ρ 0.4 mm were obtained. Standard AOAC [1990] methods were applied to evaluate chemical composition of lentil meal (Table 1).

Chemicals

Chymotrypsin (EC 3.4.21.1), L-leucine, sodium dodecyle sulfate (SDS), glycine, acrylamide, N,N'-methylene-bis-acrylamide, 2-mercaptoethanol, SDS-PAGE marker (SigmaMarker™ Low Range) and SE-HPLC standards were obtained from Sigma-Aldrich Co. Ltd. (Poznań, Poland). 2,4,6-trinitrobenzene sulphonic acid, Coomassie Brilliant Blue R-250 were purchased from Serva (Heidelberg, Germany), HPLC solvents from Merck (Darmstad, Germany). Other reagents, all of analytical grade, were acquired from P.O.Ch. Company (Gliwice, Poland).

Kinetics of hydrolysis controlled using pH-stat method

Lentil meal (3 g) was weighed into a thermostated vessel and suspended in 28 mL of distilled water. After stirring the vessel was set in a TTT 80 Titrator (Radiometer). Suspension pH was adjusted to 8.0 using 0.2 mol/L NaOH and the temperature was set at 50°C. Then chymotrypsin (50 mAU/g of protein) dissolved in 2 mL of distilled water was added. Enzyme activity evaluated with the Anson method was 1.66 AU/g [Mejbaum-Katzenellenbogen & Mochnacka, 1969]. During hydrolysis pH of 8.0 was maintained by the addition of 0.2 mol/L NaOH from a burette. The amount of the base added was recorded after 2, 5, 10,… 40, 50,… and 120 min of the process.

Degree of hydrolysis

The degree of hydrolysis (DH), which is the percentage of peptide bond cleaved, was calculated according to equation (1) [Adler-Nissen, 1986]:

$$DH = B \times N_b \times \frac{1}{\alpha} \times \frac{1}{M_P} \times \frac{1}{h_{tot}} \times 100\% \qquad (1)$$

where: B – base consumption (mL), N_b – molarity of the base (mol/L), α – average degree of dissociation of the α–NH_2 groups, M_b – mass of protein (g), and h_{tot} – total number of peptide bonds in the protein substrate (meqv Leu-NH_2/g protein).

In equation (1) a value of 1.15 was applied as α^{-1} [Adler-Nissen, 1986], whereas total number of peptide bonds in lentil meal proteins (h_{tot}) was determined after acidic hydrolysis (6 mol/L HCl at 105°C for 12 h) [Hajos *et al*., 1988] using the spectrophotometric method with 2,4,6-trinitrobenzene sulphonic acid (TNBS) [Panasiuk *et al*., 1998].

TABLE 1. Chemical composition of lentil meal.

Compound	Content (g/100 g)*
Crude protein (*N* × 6.25)	26.8 ± 0.10
Starch	47.9 ± 0.91
Lipids	0.84 ± 0.03
Ash	3.16 ± 0.07
Moisture	6.82 ± 0.15

* Data are expressed as means ± the standard deviation (n = 3).

Preparation of protein hydrolysates

In order to obtain hydrolysates of specified degree of hydrolysis, the hydrolysis was carried out under the same conditions to those applied during kinetic studies. The process was controlled with the pH-stat technique [Adler-Nissen, 1984]: after using NaOH volume resulting in DH value of 2%, 4%, 8% and 12%, the reactive mixture was heated at 90°C for 5 min in order to inactivate the enzyme. Then preparations were chilled, neutralised with 0.2 mol/L HCl, and freeze dried.

SDS-PAGE

Lentil meal proteins and their hydrolysates were denaturised by heating at 100°C for 5 min in 0.0625 mol/L Tris-HCl buffer (pH 6.8) with 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue [Hames, 1990]. Electrophoretic separation of samples was performed in 15% polyacrylamide gel in the Laemmli's buffers at a constant current of 25 mA using Mini-PROTEAN® system (BioRad). SigmaMarker™ Low Range of 6,500–66,000 Da molecular range was utilised as a standard. Quantity One Software (BioRad) was used to estimate molecular weights of resolved proteins.

SE-HPLC

Lentil meal proteins and their enzymatic hydrolysates were analysed using a Shimadzu HPLC system consisting of LC-10AD$_{vp}$ pumps, UV-VIS SPD-M10A$_{vp}$ photo-diode array detector, SCL-10A$_{vp}$ system controller. The samples were injected into a TSK Gel G2000SW$_{XL}$ (5 μm, 7.86 x 300 mm, TosoHaas) column and eluted with 45% (v/v) acetonitrile containing 0.1% (v/v) trifluoracetic acid (TFA), with a flow rate of 0.2 mL/min. The injection volume was 100 μL and the detector was set at 220 and 280 nm. Bovine serum albumin (66,000 Da), cytochrom C (12,400 Da), bovine lung aprotinin (6,500 Da), bovine insulin chain B oxidized (3 496 Da), human angiotensin II (1 046 Da), leucine enkephalin (556 Da) and Thr-Tyr-Ser (369 Da) were used as molecular weight standards.

Sensory evaluation

Aqueous solutions of lentil meal and its chymotryptic hydrolysates were prepared at a concentration of 1% (w/v). Samples of 10 mL in disposable containers were coded individually and subjected to evaluation at random order to the panelists. Filter water at room temperature was supplied as a neutralising agent between samples. The intensity of bitterness and astringency sensation were determined by the method of sensory scaling [PN-ISO 4121, 1998]. A 10 cm linear scale anchored "none" (0) to "very intensive" (10) was used. The results were expressed as arbitrary units (0-10 a.u.).

The assessment was carried out by four trained and experienced panelists, in a sensory laboratory room, following general requirements for sensory testing conditions.

RESULTS AND DISCUSSION

Crude protein content of lentil meal subjected to the present study was estimated using the Kjeldahl method ($N \times 6.25$) and was 26.8 g/100 g (Table 1). This value was within the range reported by Wang & Daun [2006]. The authors reported that crude protein content of four different lentil cultivars varied from 24.3% to 30.2% with a mean of 27.2%. Albumin and globulin are predominant fractions of lentil proteins. Bhatty [1988] noticed that albumins and globulins represented 19.6% and 53.9% of the solubilised proteins of lentil. Neves & Lourenco [1995] reported slightly lower values, 11% and 42% respectively. Other protein fractions of lentils are prolamins and glutelins.

Kinetics of hydrolysis

The curve of chymotryptic hydrolysis of lentil meal proteins, *i.e.* plot of degree of hydrolysis (DH) *vs.* time, is depicted in Figure 1. The process of hydrolysis were characterised by a high rate of the first phase, then the rate was slowed and after approximately 90 min became constant. Similar plots of hydrolysis were obtained when proteins of other plants *i.e.* soy [Seo *et al.*, 2008], pea [Karamać *et al.*, 1998; 2002], rapeseed [Chabanon *et al.*, 2007], corn [Apar & Ozbek, 2007], wheat [Kong *et al*., 2007] and milk proteins [Margot *et al*., 1997; Wróblewska *et al*., 2004] were treated by proteases. Numerous attempts were made to explain the decreasing rate of hydrolysis with time [Constainides & Adu-Amankwa, 1980; Bombara *et al*., 1992; Gonzalez-Tello *et al*., 1994]. Among the most frequently considered reasons are: decreasing in time number of polypeptide bonds prone to protease used, inhibition of enzyme activity by resulting products of hydrolysis, and enzyme denaturation.

In our studies, the final DH value obtained after 120 min of hydrolysis of lentil proteins was 13%. Chymotrypsin, a highly specific enzyme, allows for limited hydrolysis only. DH obtained is comparable to results reported by other authors who hydrolysed plant proteins using single, isolated endopeptidases [Rozan *et al*., 1997; Karamać *et al*., 2002; Kong *et al*., 2007; Ma *et al*., 2008]. Chymotryptic hydrolysis of the isolated globulin G1, major storage protein of lentil, resulted in DH 9.3%, however, when proteins were denaturated before proteolysis, DH achieved increased to 13.4% [Neves & Lourenco, 1995].

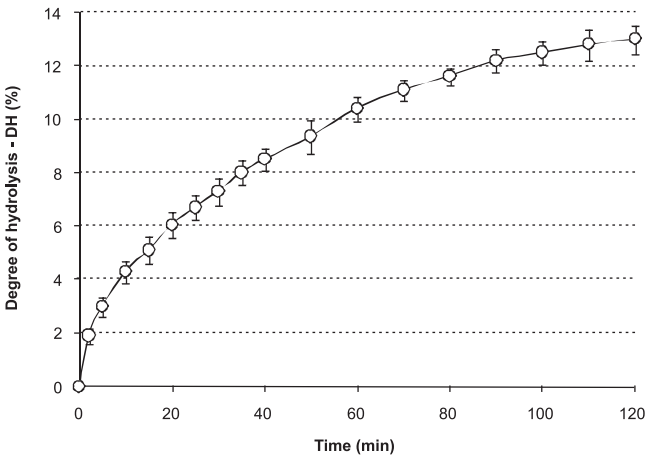

FIGURE 1. Kinetics of lentil meal proteins hydrolysis by chymotrypsin controlled using the pH-stat technique.

SDS-PAGE

Figure 2 depicts the electrophoretic separation of lentil meal proteins and their chymotryptic hydrolysates. Lentil meal proteins (DH 0%) were separated into numerous bands in the range of 21,000-66,000 Da molecular weight, two bands corresponding to higher molecular weights: 87,000 and 79,000 Da, and four weak bands in the range of 14,200-17,200 Da. Bands corresponding to proteins of molecular weight lower than 14,200 Da were not detected. Chymotryptic hydrolysis caused disappearing of some bands, the number of bands which disappeared was observed to increase along with the increase of DH. The intensity of some bands (corresponding to molecular weight of 46,000; 34,500; 22,700 Da) decreased with the increase of DH but they did not disappear, even in the case of a hydrolysate with DH 12.0%. Some new bands appeared on the electrophoregram of the hydrolysate with DH 2% and their intensity was increasing along with an increase of DH. Among those products of hydrolysis, both high molecular weight proteins and polypeptides (62,000; 30,500 Da) and peptides with much lower molecular weight (<6,500 Da) were noticed.

Worthy of mention are bands corresponding to molecular weights of 19,000 Da and approximately 8,500 Da which appear in the hydrolysate with DH 2.0% but their intensity was decreasing along with an increase of DH, and they disappeared

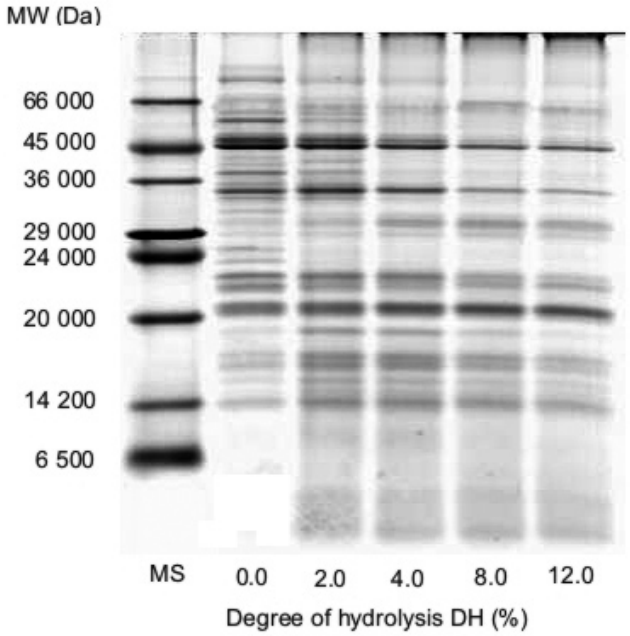

FIGURE 2. SDS-PAGE separation of lentil meal proteins and their chymotryptic hydrolysates with a different degree of hydrolysis (DH); MS – molecular weight standards, MW – molecular weight.

in the hydrolysate with DH 12.0%. Similarly, bands in the range of 14,200-17,200 Da are more intense at the initial stage of hydrolysis (DH 2%) and then fade along with the increase of DH. It can be supposed that chymotryptic hydrolysis of lentil proteins is carried out according to a "zipper" mechanism contrary to the "one-by-one" mechanism [Adler-Nissen, 1986].

Two subunits of lentil proteins with molecular weights of 22,000 and 20,500 Da were noticed to be resistant to chymotrypsin activity. Neves & Lourenco [1995] hydrolysed lentil G1 globulin and reported the presence of subunits with 32-34 kDa and 21 kDa molecular weight resistant to chymotrypsin hydrolysis.

SE-HPLC

Molecular weight distribution of lentil meal proteins and their chymotryptic hydrolysates were analysed using size exclusion HPLC with detection at 220 and 280 nm (Figures 3 and 4). Chromatograms obtained with detection at 220 nm show that molecular weight of most of lentil proteins is ≥66,000 Da, although proteins with molecular weight in the range of 12,400-66,000 Da are also present. Hydrolysis carried out to achieve DH 2% caused loss of the largest proteins and production of polypeptides with molecular weight of 6,500-12,400 Da, mainly. Then, along with the increase of DH to 4%, polypeptides and peptides with molecular weight

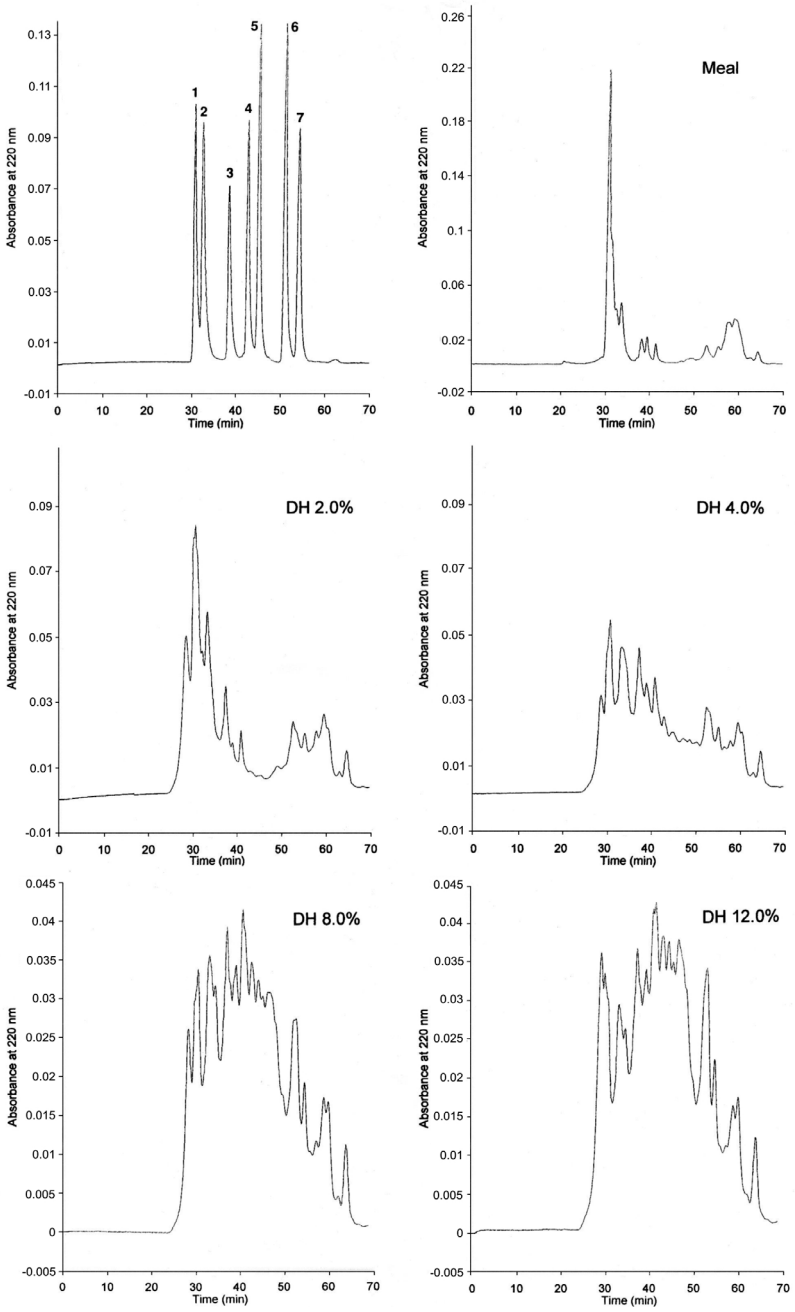

FIGURE 3. SE-HPLC chromatograms of lentil meal proteins and their chymotryptic hydrolysates with a different degree of hydrolysis (DH) detected at 220 nm. Molecular weight standards are denoted by numbers: 1 – bovine serum albumin (66,000 Da), 2 – cytochrom C (12,400 Da), 3 – bovine lung aprotinin (6,500 Da), 4 – bovine insulin chain B oxidized (3,496 Da), 5 – human angiotensin II (1,046 Da), 6 – leucine enkephalin (556 Da), and 7 – Thr-Tyr-Ser (369 Da).

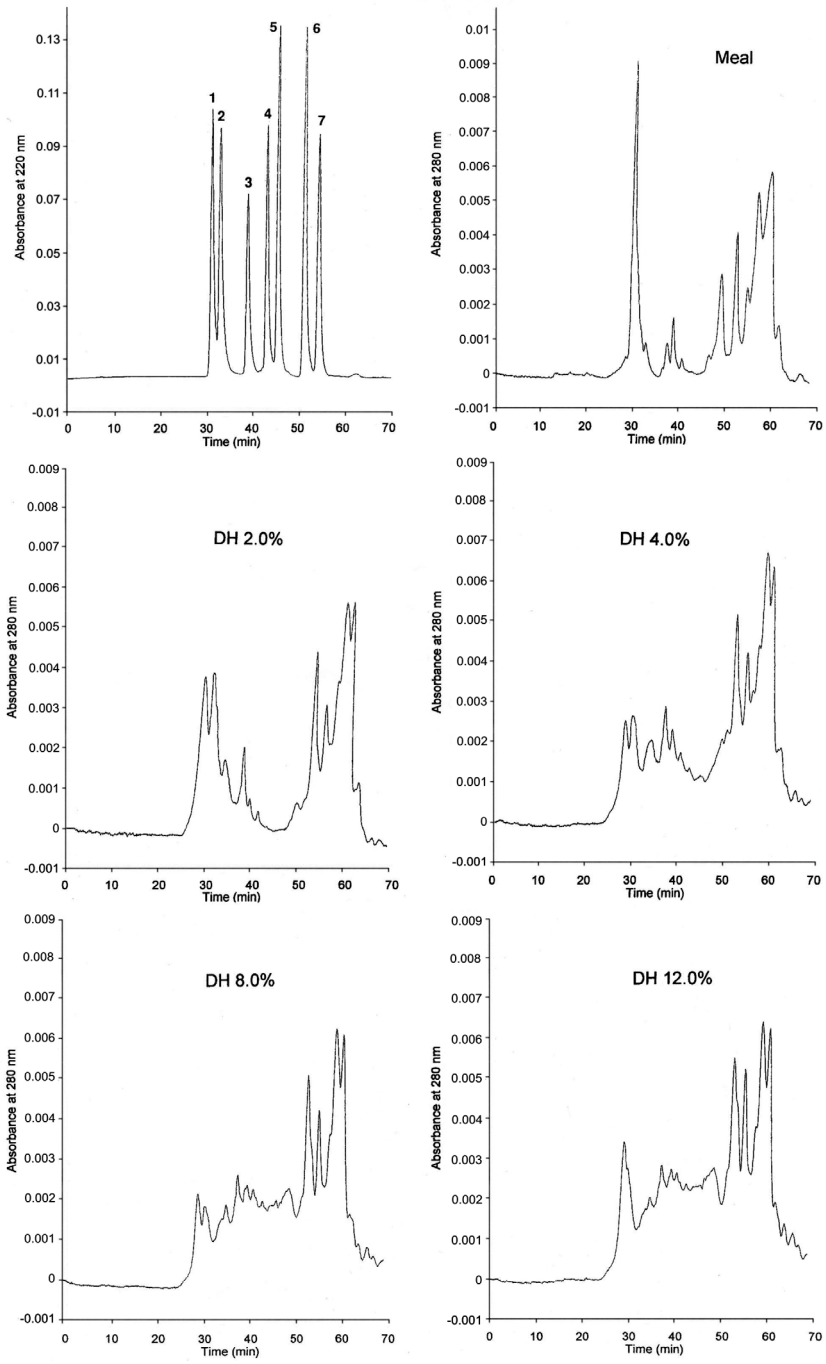

FIGURE 4. SE-HPLC chromatograms of lentil meal proteins and their chymotryptic hydrolysates with a different degree of hydrolysis (DH) detected at 280 nm. Molecular weight standards are denoted by numbers 1-7 as in Figure 3.

of 3,495–6,500 Da appeared. Hydrolysates of DH 8% and 12% contain peptides of molecular weight of 556–3,495 Da, but also polypeptides (3,495-6,500 Da), and unhydrolysed proteins (Figure 3). Figure 4 depicts SE-HPLC chromatogram recorded at 280 nm. Besides peaks originating from proteins and peptides, four high peaks from low molecular weight (<556 Da) compounds are present, either in chromatograms of lentil meal or hydrolysates. These peaks do not originate from peptides, because their absorption at 220 nm is very low. Most probably they come from phenolic compounds present in lentil meal which absorbs strongly at 280 nm. Among phenolic compounds present in lentil seeds Lopez-Amoros *et al.* [2006] identified (+)-catechin and phenolic acids *i.e.* protocatechuic, *p*-hydroxybenzoic, vanillic, trans *p*-coumaric, trans ferulic acids. In turn, Wang & Daun [2006] report on the presence of condensed tannins (0.40 – 1.01% depending on cultivar).

Sensory evaluation

Intensity of bitterness and astringency sensation was assessed, *i.e.* main attributes which may develop undesirable taste of hydrolysates.

Figure 5 depicts results of astringency sensation and bitterness intensity of lentil meal proteins and their hydrolysates

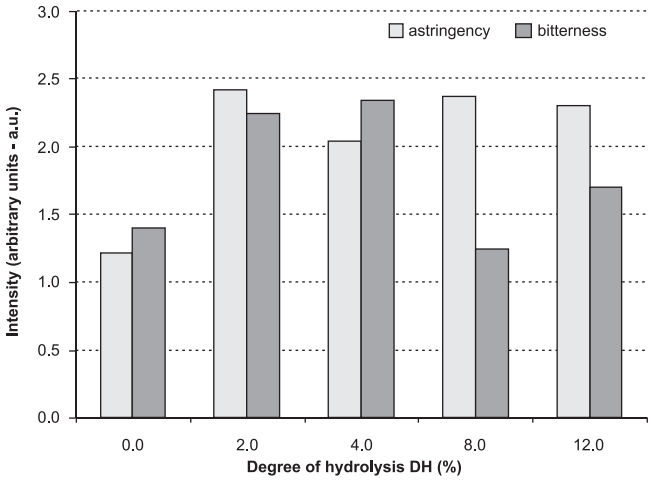

FIGURE 5. Intensity of bitterness and astringency sensation of lentil meal proteins and their chymotryptic hydrolysates with a different degree of hydrolysis (DH).

obtained using chymotrypsin. No simple correlation between degree of hydrolysis and intensity of the assessed attributes was noticed. Chymotryptic hydrolysis evoked doubled intensity of astringency sensation in comparison to the unhydrolysed sample. For all chymotryptic hydrolysates similar results were obtained, *i.e.* 2.05-2.43 a.u. Bitterness evoked during hydrolysis was higher for the hydrolysate with DH 2% (2.25 a.u.) and 4% (2.35 a.u.), and slightly higher for that with DH 12% (1.70 a.u.) whereas lower for the hydrolysate with DH 8% (1.25 a.u.) in comparison to lentil meal.

Literature data reports that bitterness of hydrolysates intensifies along with a degree of hydrolysis. This dependence was noticed for soy proteins treated with Alcalase, Neutrase, Protamex, papain and bromelain [Adler-Nissen, 1984; Seo *et al*., 2008] and casein degraded by proteases from *Aspergillus sp., Bacillus licheniformis* and *Bacillus subtilis* [Vegarud & Langsrud, 1989; Slattery & Fitzgerald, 1998]. Contrary to that, in the present study, hydrolysates with a lower degree of hydrolysis (2% and 4%) showed the most intense bitterness. It can ensue from the specificity of enzyme used. Chymotrypsin cleaves peptide bonds of tyrosine, phenylalanine and tryptophan, so 'attacks' directly the hydrophobic region of proteins and a lot of hydrophobic peptides developing bitterness are released to the solution.

However, it should be mentioned that bitterness of hydrolysates revealed in the present studies at the level of 2.25-2.35 a.u. in a 10-point scale is not high. Chymotryptic hydrolysates of pea proteins are comparably bitter (approximately 4.5 on 15-cm lines). On the other hand, bitterness of pea protein hydrolysates obtained using trypsin, Flavourzyme and Alcalase is much higher [Humiski & Aluko, 2007].

The increase of astringency intensity of chymotryptic hydrolysates of lentil meal proteins in comparison to intact proteins can be caused by the release of astringent peptides during hydrolysis. The presence of those peptides was reported in Chedder cheese [Harwalkar, 1972]. However, more probable explanation is that astringency of lentil hydrolysates was due to phenolic compounds presence in lentil meal, especially tannins. Amarowicz *et al.* [2004] reported that astringency of a phenolic compound extract of lentil seeds expressed as Sensation Astringency Indices amounts to 5.71 arbitrary units and noticed a correlation (r=0.787) between astringency and tannin content of legume seeds extract. It is well known that tannins form complexes with proteins easily. Probably some of tannins present in lentil meal were bound to proteins. It seems that the increase of astringency of the studied hydrolysates in comparison to intact proteins could result from the release of tannins from complexes during hydrolysis of proteins.

CONCLUSIONS

1. Chymotrypsin gave rise to limited hydrolysis of lentil meal proteins. Degree of hydrolysis (DH) after 120 min of the process amounted to 13%.

2. Chymotryptic hydrolysis of lentil proteins was carried out according to a "zipper" mechanism. At the first stage of hydrolysis, intermediate products were formed, which were then further hydrolysed.

3. Chymotrypsin treatment resulted in releasing of hydrolysis products of both high molecular weight molecules and small peptides. Larger polypeptides and unhydrolysed proteins were present in the hydrolysate with DH 12% but products of hydrolysis with molecular weight of 0.5–6.5 kDa were predominant.

4. No simple correlation was noticed between a degree of hydrolysis and intensity of bitterness and astringency sensation. Bitterness of hydrolysates, at the level of 2.25-2.35 a.u. in a 10-point scale was not high. Astringency of hydrolysates was due to phenolic compounds presence in lentil meal.

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