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Preparation of reductive polypeptides from fresh placentas of dairy cows

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Abstract

In order to prepare reductive polypeptides from the placenta of dairy cows' fresh placentas from healthy Chinese Holstein cows were obtained and homogenized. Response surface model was established to optimize the hydrolysis condition for the extraction of the placental polypeptides. Specifically, the placental tissue homogenate was treated with both trypsin and pepsin for 348 min and 329 min; at 35.00% and 35.75% of substrate concentration; with an enzyme-substrate ratio of 3.33% and 3.92%, respectively, based on the models. The treated samples were then demineralized and freeze-dried to obtain the hydrolyzed polypeptides. In order to identify the molecular mass distribution and reducibility of polypeptides, matrix-assisted laser desorption ionization (MALDI) and Prussian blue methods were used. The concentrations of placental polypeptides after hydrolysis by trypsin or pepsin were 5.52% and 5.97%, respectively; the vitamin C (Vit C) equivalents were 36.26 μg mg^{-1} or 61.15 μg mg^{-1}, respectively. Both groups showed intensity peaks of MALDI patterns in the range of 300 - 400 Da, and polypeptides hydrolyzed by pepsin had higher Vit C equivalent anti-oxidant activity than trypsin hydrolyzed polypeptide, suggesting that the proteins in the placental tissues were hydrolyzed to di-peptides and tri-peptides completely. In conclusion, both trypsin and pepsin hydrolysis performed well in preparation of reductive polypeptides from the fresh placentas of dairy cows; while, pepsin is more effective than trypsin. The primary reductive ingredients may be the oligopeptides with molecular mass less than 1000 Da.

Introduction

Peptides from animal and plant tissues were widely investigated due to their nutritional values and multiple bioactivities such as anti-oxidant, anti-inflammatory, anti-hypertensive, pro-immune and endocrine functions both *in vitro* and *in vivo*.[1-3] Moreover, previous studies have found that these active peptides are predominantly the hydrolysates from inactive proteins.[4-6] Some food-derived bioactive peptides are produced through the hydrolysis of ingested protein. Furthermore, these peptides could be absorbed directly because of their small molecular mass and simple structures to exhibit their bioactivities.[7] Some bioactive peptides can also be derived from wastes in the breeding and food industry, like animal bones and placentas, using appropriate hydrolysis protocols.[8] The placenta is a temporary organ in the uterus during gestation; but, plays a vital role in fetal development and endocrine regulations.[9] Placenta contains several kinds of bio-active molecules, including hormones, proteins and lipids, and there have been many researches showing that the hydrolysates of placenta exhibit multiple functions towards the anti-aging[10] non-alcoholic fatty liver disease,[11] wound-healing,[12] etc. Unfortunately, placentas are often discarded after parturition. In order to establish protocols to prepare reductive polypeptides from the placenta of dairy cows, we used trypsin and pepsin to hydrolyze the placenta of dairy cows; then, the molecular mass distribution of peptides in hydrolysates was determined using matrix-assisted laser desorption ionization (MALDI). We also measured the reducing power of peptides using Prussian blue method. The study here established a novel protocol for efficient extraction of polypeptides from dairy cows placentas.

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Materials and Methods

Materials. After normal parturition, the placentas from healthy Chinese Holstein cows (located at a commercial dairy farm in Sichuan Province, China, with weight around 600 kg and 2 - 4 parity) were collected and rinsed with normal saline having 4.00 ˚C temperature immediately to remove the blood and dirt and subsequently stored in polythene bags at – 20.00 ˚C till their further usage.

Test equipments. The HH-4 digital display constant temperature water bath (Changzhou Guohua Electric Co., Ltd. Jiangsu, China); LYOQUEST freeze dryer (Azbiler Telstar Mechanical and Electrical Equipment Co., Ltd. Shanghai, China); electrophoresis system (GE Healthcare Bio-Science, Uppsala, Sweden); Sorvall™ Legend™ XT refrigerated centrifuge (Thermofisher Scientific Co., Ltd. Shanghai, China); Varioskan LUX full-wavelength multimode microplate reader (Thermofisher Scientific Co., Ltd.); Barnstead™ GenPure™ Pro water purifier (Thermofisher Scientific Co., Ltd.); Q Exactive mass spectrometer (Thermofisher Scientific Co., Ltd.); Easy nLC chromatography system (Thermofisher Scientific Co., Ltd.).

Test reagents. Trypsin (250 U mg^{-1}); Pepsin (250 U mg^{-1}); Potassium dihydrogen phosphate (KH_2PO_4); Di-sodium phosphate (Na_2HPO_4); Trichloroacetic acid (Cl_3COOH); Ferric chloride ($FeCl_3$); Potassium ferricyanide ($K_3Fe(CN)_6$); Vitamin C (Vit C; were purchased from Chengdu Kelong Chemical Co., Ltd. Chengdu, China); and C18 Cartridge (Sigma, St. Louis, USA.); Formic acid (FA) (Fluka, Seelze, Germany); Bovine serum albumin (BSA; Sangong Co. Ltd. Shanghai, China) were used.

Homogenization of cow's placental tissue. Frozen placental tissues were weighed, cut to small pieces and then put into a beaker, after this they were heated at 37.00 ˚C in the water bath. They were shredded with scissors and then homogenized at 12,000 rpm in the ice bath until no tissue fragments were observed.

Response surface modelling. Hydrolysis conditions were designed on the basis of Box-Behnken Center Response by design expert 8.0.6, selected hydrolysis time (A), substrate concentration (B) and enzyme/substrate mass ratio (E/S; C) as factors into three factors and three levels test design, with total 17 test points including five central and 12 factorial points. The level design referenced to goat placenta enzymatic conditions. Levels -1, 0 and 1 of factors A, B and C respectively stand for 180 min, 300 min and 420 min of hydrolysis, 10.00%, 30.00% and 50.00% of substrate concentration and 1.00%, 3.00% and 5.00% of E/S.

Hydrolysis. Enzymatically hydrolyzed placental homogenates strictly followed optimum conditions of temperature and pH (37.00 ˚C, pH: 8.00 for trypsin, and 37.00 ˚C, pH: 2.50 for pepsin). Then, they were hydrolyzed under the optimal condition of response surface model. After that, they were heated at 100˚C for 10 min to inactivate enzymes in the water bath. Hydrolyzed liquid was centrifuged at 6,000 rpm for 10 min, the supernatant was collected and freeze-dried for 24 hr, the freeze-dried powder was obtained as placental hydrolysates, the hydrolysates were weighed and then the extraction rate (R) was calculated as:

$$R\ (\%) = 100 \times \text{hydrolysates mass/homogenized placental mass}$$

Matrix-assisted laser desorption ionization analysis of polypeptides. Hydrolysates were mixed by the test tubes number (1-4, 2-5 and 3-6) separately in both of trypsin and pepsin groups. Then, they were demineralized by C18 Cartridge in chromatography system, freeze-dried and dissolved in 1.00 mL of 0.10% FA solution to measure the protein concentrations by spectrophotometer under OD_{280}. After this, certain molecular mass of peptides was determined using MALDI.

Reducibility measurement. This study applied the Prussian blue method to measure the reducing power of placental hydrolysates. The reductive efficiency was measured by conversion of potassium ferricyanide to potassium ferrocyanide ($K_4Fe(CN)_6{\cdot}3H_2O$) with the help of these hydrolysates, and production of Prussian blue ($Fe_4[Fe(CN)_6]_3$) by the addition of $FeCl_3$ to potassium ferrocyanide solution. Prussian blue has an absorbance peak at 700 nm (A_{700}), and the value of A_{700} showed a linear correlation to the Prussian blue concentration. Our study used 100, 50, 25, 12.50, 6.25 and 3.125 μg mL^{-1} of Vit C solution to establish the standard curve. The 10.00% trichloroacetic acid solution, 1.00% potassium ferricyanide solution, 0.10% ferric chloride solution and phosphate buffer solution (pH: 6.60) were also prepared for this purpose. In 75.00 μL of each standard solution, 75.00 μL of phosphate buffer and 1.00% potassium ferricyanide solution were infused separately, oscillated and heated at 50.00 ˚C in the water bath for 20 min. The 75.00 μL of 10.00% trichloroacetic acid solution was injected subsequently and then centrifuged at 3,000 rpm for 1 min to prepare the test solution. The 200 μL of each test solution was transfused to a 96-well plate, then, 20.00 μL of ferric chloride solution was injected subsequently and A_{700} was measured. Standard curve was established by linear regression equation in SPSS (version 24.0; IBM Corp., Armonk, USA), with Vit C concentration of standard solutions as the abscissa and their A_{700} as the ordinate. Placental hydrolysates of each group were dissolved to 1.00 mg mL^{-1} sample solution and then, A_{700} was measured according to the protocol above. The A_{700} of each sample solution was substituted to the standard curve to acquire the reducibility of polypeptides being shown as Vit C equivalent (μg mg^{-1}) of the placental hydrolysates.

Statistical analysis. The data of extraction rate and reducibility were analyzed by SPSS (IBM Corp.) to distinguish the significant differences between the hydrolysates of trypsin and pepsin.

Results

Response surface modelling of trypsin hydrolysis. Hydrolysis of the placental homogenates was done under the optimum conditions of the temperature and pH (pH: 8.00 and 37.00 ˚C). The actual yield, substrate and enzyme mass were shown in Table 1. When factor A was -1, B was 0 and C was -1 and 1 and also, A was -1, C was 0 and B was -1 and 1, the Rs were 4.39%, 5.70%, 4.03% and 5.02%, respectively. When factor A was 0, B was -1 and C was -1 and 1 and also, A was 0, B was 1 and C was -1 and 1, the Rs were 4.00%, 5.71%, 5.73% and 6.45%, respectively. When factors A, B and C were 0, the Rs were 6.78%, 6.31%, 6.45%, 6.18% and 6.04%, respectively. When factor A was 1, B was 0 and C was -1 and 1 and also, A was 1, C was 0 and B was -1 and 1, the Rs were 5.28%, 6.80%, 5.41% and 6.34%, respectively. Response surface regression equation of R was as follows:

$$R = 5.50 + 0.15A + 0.026B + 0.38C – 0.8AB - 0.075AC + 0.13BC – 0.18A^2 – 0.54B^2 – 1.46C^2$$

Table 2 shows the regression analysis results of both models; both models were significant ($p < 0.01$), no significance lack of fit was found ($p > 0.05$), R^2 was 98.85% and adjustment R^2 was 97.37%. The model showed considerable demonstration and reliability in the statistical analysis. Variances A, C, B^2 and C^2 significantly influenced the R ($p < 0.01$). The F value showed influence rank among the factors as C > A > B. Figure 1 shows the R plateaued as hydrolysis time increased, indicating that the enzymatic reaction was almost complete at the designed levels (Figs. 1A and 1B); the R showed a parabola-like trend with increasing E/S; but, test groups with higher E/S still showed higher R in the entire test, which may be caused by self-hydrolysis reaction in high enzyme concentration (Fig. 1C). Optimized levels of all factors to maximize the R and reducing capability were based on the response surface model separately. The optimized enzymatic hydrolysis conditions were hydrolysis time of 346 min, substrate concentrations of 30.21% and E/S of 3.24% and the R was 5.55%. We performed three repetition tests under the optimized conditions to verify the reliability of both models; the mean actual hydrolysis time was 345 min, mean substrate concentration was 30.00%, mean E/S was 3.00% and mean actual R was 5.48%, which were consistent with the theoretical value.

Response surface modelling of pepsin hydrolysis. The placental homogenates were hydrolyzed per the instructions (pH: 2.50 and 37.00 ˚C). Actual yield, substrate and enzyme mass were shown in Table 1. When factor A was -1, B was 0 and C was -1 and 1 and also, A was -1, C was 0and B was -1 and 1, corresponding Rs were 2.97, 4.47, 3.23 and 3.73%, respectively. When factor A was 0, B was -1 and C was -1 and 1 and also, A was 0, B was 1 and C was -1 and 1, corresponding Rs were 5.03%, 5.25%, 5.32% and 5.88%, respectively. When factors A, B and C were 0, corresponding Rs were 5.92%, 5.81%, 5.84%, 6.03% and 5.57%, respectively. When factor A was 1, B was 0 and C was -1 and 1 and also, A was 1, C was 0 and B was -1 and 1, corresponding Rs were 5.38%, 5.30%, 5.67%, and 5.84%, respectively. Response surface regression equation of R was as follows:

$$R = 5.83 + 0.97A + 0.29B + 0.18C – 0.4AB-0.083AC + 0.085BC – 1.03A^2 – 0.28B^2 – 0.19C^2$$

Table 2 shows the regression analysis of both models, which were significant ($p < 0.01$); no significant lack of fit was found ($p > 0.05$), R^2 was 97.54% and adjustment R^2 was 94.38%. The model showed remarkable reliability in the statistical analysis. Variances A, B, A^2, and AC showed very significant effects on the R ($p < 0.01$). The F value showed influence rank among the factors as A > B > C. Figure 1 shows a parabola-like trend between the Rand time under a certain E/S value (Fig. 1D); the same trend was also observed with the change of E/S ratio under a certain hydrolysis time (Fig. 1E). Under a certain substrate concentration, R increased with the E/S ratio, and showed a negative correlation with the hydrolysis time (Fig. 1F). Optimized levels of all factors to maximize the Rand reducing capability were based on the response surface model separately. The optimized enzymatic hydrolysis conditions were hydrolysis time of 373 min, substrate concentrations of 30.71% and E/S of 3.18% and corresponding R was 6.14%. We performed three repetition tests under the optimized conditions to verify the reliability of both models; the mean actual hydrolysis time was 378 min, mean substrate concentration was 30.00%, mean E/S was 3.00% and mean actual R was 6.05%, which were close to the theoretical value.

Extraction of polypeptides in placental hydrolysates. The mean Rs of trypsin and pepsin hydrolysates were 5.48% and 6.04%, respectively (Table 3). After chromatographic demineralization, the protein concentrations of trypsin and pepsin hydrolysates were 95.00%, 95.56% and 94.17%, and 94.02%, 96.08% and 95.46%, respectively (Table 4).

Table 1. Response surface of trypsin and pepsin hydrolyzed placenta polypeptides.

Groups	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
A	-1	-1	1	-1	0	1	1	0	0	0	1	0	0	0	0	0	-1
B	0	0	-1	-1	1	0	1	-1	0	0	0	0	0	1	0	-1	1
C	1	-1	0	0	1	-1	0	1	0	0	1	0	0	-1	0	-1	0
Trypsin extraction rate (%)	5.70	4.39	5.41	4.03	6.45	5.28	6.34	5.71	6.78	6.31	6.8	6.45	6.18	5.73	6.04	4.00	5.02
Pepsin extraction rate (%)	2.97	5.38	4.47	5.30	3.23	5.67	3.73	5.84	5.03	5.32	5.25	5.88	5.92	5.81	5.84	6.03	5.57

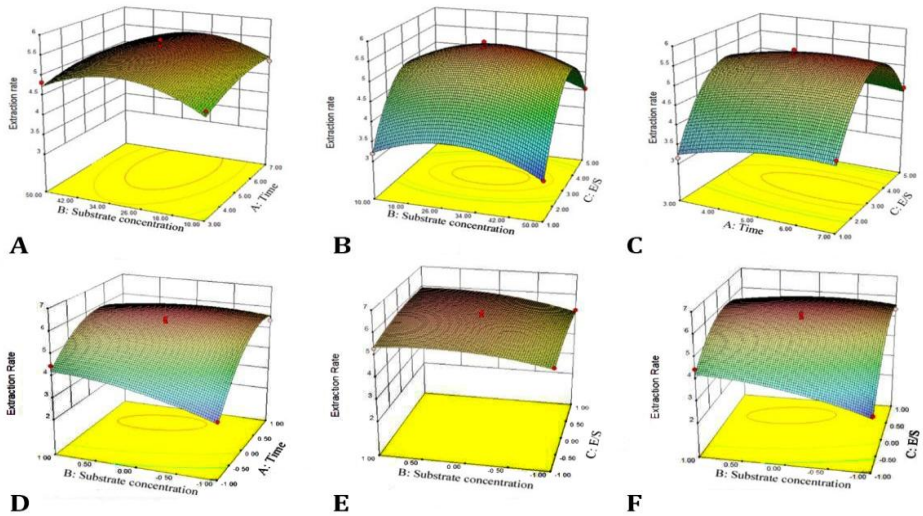

Fig. 1. Effects of various factors on extraction rate of trypsin and pepsin digested dairy cow placentas. Note: **A, B,** and **C** are factors interactions in trypsin hydrolysis and **D, E,** and **F** are factors interactions in pepsin hydrolysis.

Table 2. Regression model analysis of variance analysis of trypsin and pepsin digested placenta.

Groups	Source*	Model	A	B	C	AB	AC	BC	A^2	B^2	C^2	Residual	Lack of fit	Error	Total
	SS	13.86	0.70	0.12	0.86	0.048	0.002	0.065	0.08	0.65	10.85	0.15	0.11	0.04	14.02
	DoF	9	1	1	1	1	1	1	1	1	1	7	3	4	16
Trypsin	**MSE**	1.54	0.70	0.12	0.86	0.048	0.002	0.065	0.08	0.65	10.85	0.022	0.038	0.009 95	
	F-value	70.32**	32.05**	5.37	39.17**	2.21	0.092	2.97	3.63	29.61**	495.19**		3.80		
	***p*-value**	< 0.0001	0.0008	0.0536	0.0004	0.1807	0.7699	0.1286	0.0983	0.001	< 0.0001		0.1148		
	SS	14.03	7.59	0.64	0.14	0.62	0.027	0.0081	2.55	1.16	0.81	0.38	0.27	0.12	14.42
	DoF	9	1	1	1	1	1	1	1	1	1	7	3	4	16
Pepsin	**MSE**	1.56	7.59	0.64	0.14	0.62	0.027	0.0081	2.55	1.16	0.81	0.055	0.089	0.029	
	F-value	28.48**	138.57**	11.66**	2.52	11.40	0.50	0.15	46.59**	21.26**	14.77		3.07		
	***p*-value**	0.0001	<0.0001	0.0112	0.1566	0.0118	0.5035	0.7119	0.0002	0.0025	0.0063		0.1537		

* Source: Source of variance; SS: Sum of square, DoF: degrees of freedom, MSE: Mean square error.
** Asterisks mean very significant ($p < 0.01$), the same below.

Table 3. Verification and reducibility of placental hydrolysates modeling.

Parameters	Trypsin						Pepsin					
Number	1	2	3	4	5	6	1	2	3	4	5	6
Homogenates weight (g)	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Enzyme weight (g)	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18
Hydrolysis time (min)	345	345	345	345	345	345	378	378	378	378	378	6.30
Total volume (mL)	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Extraction rate (%)	5.50	5.43	5.52	5.46	5.48	5.49	5.98	6.03	6.10	6.05	6.08	6.06
Average (%) ± SE			5.48 ± 0.32[a]						6.05 ± 0.41[b]			
A_{700}			0.61398						0.602172			
Vitamin C equivalent (μg mg^{-1})			36.95						36.23			
Average			36.26 ± 0.35[a]						61.15 ± 0.12[b]			

[ab] Superscripts indicate a significant difference ($p < 0.01$).

Table 4. Protein concentrations of various demineralized placental hydrolysates.

Group	$Weight_0$ (mg)	$Weight_D$ (mg)	$Weight_R$ (mg)	A_{280}	Concentration (mg mL^{-1})	Protein (%)
BSA	-	-	1.00	0.703	1.00	-
Trypsin-1	7.80	7.10	1.00	0.668	0.95	95.00
Trypsin-2	8.10	7.50	0.90	0.613	0.87	95.56
Trypsin-3	7.90	7.50	1.20	0.817	1.16	94.17
Pepsin-1	9.50	8.80	1.00	0.661	0.94	94.02
Pepsin-2	8.70	8.20	1.10	0.743	1.05	96.08
Pepsin-3	8.70	7.90	0.90	0.604	0.86	95.46

BSA: Bovine serum albumin, $Weight_0$: Weight of hydrolysates before demineralization, $Weight_D$: Weight of freeze-dried powder after demineralization, and $Weight_R$: Weight resolved in formic acid.

The MALDL analysis of polypeptides in placental hydrolysates. The MALDI patterns showed that the molecular mass of trypsin hydrolysates had a uniform distribution between 200 – 10,000 Da (Table 4).

Reducibility measurement of placental reductive polypeptides. The corresponding A_{700} of standard Vit C solutions with concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 μg mL^{-1} were 1.67, 0.802, 0.367, 0.187, 0.15 and 0.097, respectively. A linear regression model was established as below:

$$A_{700} = 0.0164 \times \text{Vit C concentration} + 0.008$$

The standard errors (SE) of intercept and the independent variable were 0.0025 and 0.001, respectively, and the relevant R^2 was 99.60%, which showed a reliable prediction (Table 4). The mean Vit C equivalents of trypsin and pepsin were 36.26 and 61.15 μg mg^{-1}, respectively, and the Vit C equivalent of pepsin hydrolysates was significantly higher than that of trypsin hydrolysates ($p < 0.01$; Table 3).

Discussion

Trypsin is a gastrointestinal digesting protease, because of its specificity, it has been widely used in cell culture, proteomic research and polypeptide preparation. Trypsin has a high activity and less self-enzymatic reaction under the optimum conditions of temperature and pH (37.00 ˚C and pH: 8.00) and its substrates can be hydrolyzed effectively. Because of its high specificity, trypsin hydrolyzes particular proteins into their particular polypeptides and many trypsin hydrolysates showed bioactivities in different pathways. In addition to the food-derived proteins, a lot of studies have reported hydrolysis of the proteins (other than food-derived) using trypsin for preparation of polypeptides.[13-15] Proteins from animal sources can also be hydrolyzed into polypeptides and most of these polypeptides showed different bioactivities.[5,16,17] However, some researches have indicated that the Rand bioactivity may not always have a positive correlation.[18] Natural proteins often have a complex structure with a high molecular weight, indicating that the cleavage site may not be unique among these proteins; the peptides prepared through hydrolysis of protein could be further hydrolyzed to become smaller and shorter.[19] In our study, MALDI patterns showed that trypsin digested poly-peptides have intensity peaks from 100 Da to 10,000 Da, mainly clustered in 100 - 4,000 Da. All three groups showed the highest intensity value in 304 Da, indicating that placental proteins may have more than one cleavage sites for trypsin and can be hydrolyzed into oligopeptides. Although trypsin hydrolyzed polypeptides showed 36.26 μg mg^{-1} of Vit C equivalent as their reducibility, it is still significantly lower than pepsin hydrolysates. The molecular masses of trypsin catalysed hydrolysates distributed more dispersed than those catalyzed by pepsin and these smaller peptides or oligopeptides are the primary anti-oxidant ingredients in the polypeptides. [20]

Pepsin is an endogenous protease with more cleavage sites but less specificity compared to that of trypsin. Because of requirement of acidic environment (pH: 2.50) for the optimal activity of pepsin, it is less used for the preparation of polypeptides. However, as an essential digestive enzyme in the body, using pepsin to hydrolyze the placental tissue of dairy cows has the prospect for elucidating the bioactivity mechanism of placental polypeptides. Because of the extremely acidic optimal pH of pepsin, the substrate proteins will be denatured; hence, pepsin catalyzed hydrolysis can yield a higher R. Furthermore, pepsin can hydrolyze inactive exogenous proteins into bioactive peptides, including reductive polypeptides.[21,22] In this study, the placental pepsin hydrolysates showed significantly higher Rand Vit C equivalent compared to those of trypsin. Meanwhile, the peaks of pepsin hydrolyzed peptides were mainly in 304 Da and showed less peaks over 1,000 Da than trypsin hydrolyzed peptides, which is consistent with the hypothesis of Ma *et al.*,[20] that oligopeptides were the main anti-oxidant ingredients in the hydrolysates. This also suggested that oligopeptides with molecular mass less than 1,000 Da were the primary anti-oxidative polypeptides. Furthermore, the main reason for the higher Rand reducibility may be the less specified cleavage sites of pepsin.

The average Rs for trypsin and pepsin hydrolysates of cows placenta were 5.52% and 5.97%, respectively. The placental hydrolyzed polypeptides were distributed between 300 - 10,000 Da and showed intensity peaks in range of 300 - 400 Da, indicating that the main ingredients of the polypeptides were oligopeptides. Moreover, pepsin hydrolysates contained more oligopeptides and showed significantly higher reducibility compared to that of trypsin hydrolysates, indicating that we prepared reductive polypeptides, and among these reductive polypeptides there may be mainly oligopeptides within 1000 Da.

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Conflict of interest

The authors declare no conflict of interest with any financial organization regarding the material discussed in the manuscript.

References

1. Seppo L, Jauhiainen T, Poussa T, et al. A fermented milk high in bioactive peptides has a blood pressure–lowering effect in hypertensive subjects. Am J Clin Nutr 2003; 77(2): 326-330.
2. Korhonen H, Pihlanto A. Bioactive peptides: Production and functionality. Int Dairy J 2006; 16(9): 945-960.
3. Yousr M, Howell N. Antioxidant and ACE inhibitory bioactive peptides purified from egg yolk proteins. Int J Mol Sci 2015; 16(12): 29161-29178.
4. Paul M, Somkuti GA. Degradation of milk-based bioactive peptides by yogurt fermentation bacteria. Lett Appl Microbiol 2009; 49(3): 345-350.
5. Shazly AB, He Z, El-Aziz MA, et al. Fractionation and identification of novel antioxidant peptides from buffalo and bovine casein hydrolysates. Food Chem 2017; 232: 753-762.
6. Udenigwe CC, Aluko RE. Food protein-derived bio-active peptides: production, processing, and potential health benefits. J Food Sci 2012; 77(1): R11-R24.
7. Tuenter E, Bijttebier S, Foubert K, et al. *In vitro* and *in vivo* study of the gastrointestinal absorption and metabolisation of hymenocardine, a cyclopeptide alkaloid. Planta Med 2017; 83(9): 790-796.
8. Li Y, Jin Y, Li J, et al. Effects of thermal pretreatment on the biomethane yield and hydrolysis rate of kitchen waste. Appl Energ 2016; 172: 47-58.
9. Spencer TE, Johnson GA, Burghardt RC, et al. Progesterone and placental hormone actions on the uterus: insights from domestic animals. Biol Reprod 2004; 71(1): 2-10.
10. Yamasaki M, Hasegawa S, Takahashi H, et al. Placental extracts induce the expression of antioxidant enzyme genes and suppress melanogenesis in B16 melanoma cells. Nat Prod Res 2015; 29(22): 2103-2106.
11. Shimokobe H, Sumida Y, Tanaka S, et al. Human placental extract treatment for non-alcoholic steatohepatitis non-responsive to lifestyle intervention: A pilot study. Hepatol Res 2015; 45(9): 1034-1040.
12. Thakur G, Thomas S, Bhargava D, et al. Does topical application of placental extract gel on postoperative fibrotomy wound improve mouth opening and wound healing in patients with oral submucous fibrosis? J Oral Maxillofac Surg 2015; 73(7): 1439. e1-10. doi:10.1016/j.joms.2015.03.020.
13. Kyung Koh B, Ah-Song K. Effects of trypsin-hydrolyzed wheat gluten peptide on wheat flour dough. J Sci Food Agric 2008; 88(14): 2445-2450.
14. Saiga A, Okumura T, Makihara T, et al. Angiotensin I-converting enzyme inhibitory peptides in a hydrolyzed chicken breast muscle extract. J Agric Food Chem 2003; 51(6): 1741-1745.
15. Jost R, Monti JC. Partial enzymatic hydrolysis of whey protein by trypsin. J Dairy Sci 1977; 60(9): 1387-1393.
16. Klomklao S, Benjakul S, Visessanguan W, et al. Proteolytic degradation of sardine (Sardinella gibbosa) proteins by trypsin from skipjack tuna (Katsuwonus pelamis) spleen. Food Chem 2006; 98(1): 14-22.
17. Zeng WC, Zhang WC, Zhang WH, et al. Antioxidant activity and characterization of bioactive polypeptides from bovine hair. React Funct Polym 2013; 73(3): 573-578.
18. Sani IM, Iqbal S, Chan KW, et al. Effect of acid and base catalyzed hydrolysis on the yield of phenolics and antioxidant activity of extracts from germinated brown rice (GBR). Molecules 2012; 17(6): 7584-7594.
19. Thompson RC, Blout ER. Dependence of the kinetic parameters for elastase-catalyzed amide hydrolysis on the length of peptide substrates. Biochemistry 1973; 12(1): 57-65.
20. Ma Y, Wu Y, Li L. Relationship between primary structure or spatial conformation and functional activity of antioxidant peptides from Pinctada fucata. Food Chem 2018; 264: 108-117.
21. Garcés-Rimón M, González C, Uranga JA, et al. Pepsin egg white hydrolysate ameliorates obesity-related oxidative stress, inflammation and steatosis in Zucker fatty rats. PloS One 2016; 11(3): e151193. doi:10.1371/journal.pone.0151193.
22. Sun Q, Yongkang L. Porcine hemoglobin hydrolysate prepared with pepsin: antioxidant activities and their mechanisms. Int J Food Prop 2011; 14(4): 840-853.