Label-free proteomic comparative analysis of cow placental proteins enzymatic hydrolysis by different proteases

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Article

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Abstract

New found in biochemical characteristics of placenta can bring new insight for further studies on the possible markers of physiological/pathological pregnancy or function of placenta. We compared the proteome of dairy cow placenta enzymatic hydrolysis by different proteases by label-free mass spectrometry approach. In total 541, 136 and 86 proteins were identified in trypsin group (TRY), pepsin group (PEP) and papain group (PAP). By comparing the proteome of PAP and TRY, 432 differentially expressed proteins (DEPs) were identified. PEP vs TRY identified 421 DEPs, while 136 DEPs were identified in PEP and PAP. The results showed the proteins identified by papain are mostly derived from extracellular matrix and collagen and enriched in relaxin signaling pathway, AGE-RAGE signaling pathway in diabetic complications; pepsin digestion can identify more muscle-related proteins, which are enriched in lysosome, platelet activation, cardiac muscle contraction, bacterial invasion of epithelial cell and small cell lung cancer; trypsin mainly enzymatically degrades extracellular matrix, blood particles, and cell surface proteins which enriched in arginine and proline metabolism, olfactory transduction proteasome, protein processing in endoplasmic reticulum, pyruvate metabolism and arrhythmogenic right ventricular cardiomyopathy (ARVC). In summary, these results provide insights into the selection of protease in dairy cow placenta proteomics.

Introduction

The placenta is an organ that connects the mother and the fetus to maintain a stable environment for the growth and development of the fetus. It regulates the growth and development of the fetus by regulating the supply of nutrients, gases, hormones, etc and has substance exchange, hormone secretion and barrier effects. The placenta changes the mother’s endocrine system and immune system, establishing a blood vessel link between the mother and the embryo, which is the key to maintaining the growth of the fetus. Abnormal placental function is one of the principal causes of fetal death. Proteomic technology is a common tool for studying placental abnormalities. It can study the changes of placental protein during the disease process, clarify the pathogenesis and search for differentially expressed proteins related to the pathogenesis, providing an effective means for clinical diagnosis of the disease. Placental proteomics can diagnose mare peptides and find related biomarkers; through placental proteomics, it has been identified that differential proteins in the placenta of human preeclampsia are closely related to mitochondrial function, indicating that mitochondrial dysfunction is a precursor One of the pathogenesis of epilepsy; through placental proteomics, it is found that the occurrence of human recurrent miscarriage is closely related to the core factors of early embryonic development such as angiotensinogen (AGT), MAPK14 and prothrombin (F2). The digestion and extraction of protein by protease is an important factor affecting the results of proteomics. At present, trypsin is often used in placental proteomics for protein extraction. Trypsin has the advantage of high specificity, only cutting arginine (R) and lysine (K) residues, but some proteins lacking arginine and lysine residues cannot be digested by trypsin. In addition, when the trypsin cleavage site is located after the glycosylated asparagine, the attached carbohydrates may sterically prevent trypsin cleavage. In order to overcome the limitations of trypsin,
use non-specific enzymes, such as papain and pronase to digest the protein, which can completely digest the protein. Trypsin is most effective in a neutral or slightly alkaline environment, but some proteins have low solubility in this environment and cannot be digested. Pepsin has the strongest activity in an acidic environment and can enzymatically hydrolyze proteins with greater solubility in acidic environment. In this experiment, based on the Box-Behenken central response method, the optimal enzymatic hydrolysis of cow placental peptides trypsin, pepsin and papain with high reducing activity and extraction rate has been established, and the label-free technology is used to hydrolyze the above protease. Qualitative and quantitative analysis of the protein and peptides obtained from cow placenta is to compare the biological information of cow placenta hydrolyzed by different proteases, which provides theoretical support for the study of protease selection in cow placenta proteomics.

**Material And Methods**

**Ethics statement**

Sample collection was performed in strict accordance with the guidelines of the Care and Use of Laboratory Animals of China, and all procedures were approved by the Animal Care and Use Committee of Sichuan Agricultural University.

**ARRIVE statement**

This study was carried out in compliance with the ARRIVE guidelines.

**Placenta collection and preparation**

Placental samples were collected from 9 healthy pregnant Chinese Holstein cows in a large-scale semi-closed unified farm in Sichuan province. The cows were selected according to the following criteria, aged between 3–5 years, with body weight above 600 kg, 2-4 parity and around 40 weeks of pregnancy. The placenta was collected after nature delivery. Only similar parts of the placentome were used for further analysis. Wash placenta in cold saline, then portion, freeze and store it at -20°C.

Prepare placenta extract according to the method reported in Shen et al. Homogenize placenta by homogenizer (FSH 2A homogenizer; Yuexin, China) in ultrapure water, then divided into three groups contains three placenta homogenizations from three cows. Enzymatically digested homogenates under optimal conditions: trypsin (EC 3.4.21.4), pepsin (EC 3.4.23.1) and papain (EC 3.4.22.2) enzymatic dissociation at reaction time 5.80h, 4.70h, 5.49h, substrate concentration 34.96%, 34.03%, 35.74% and enzyme base ratio 3.33%, 3.66%, 3.92%, respectively. Each enzymatic hydrolysis was repeated 3 times. They are respectively marked as trypsin group (TRY, include trypsin-1, trypsin-2, trypsin-3); papain group (PAP, include papain-1, papain-2, papain-3) and pepsin group (PEP, include pepsin-1, pepsin-2, pepsin-3). After digestion, homogenates were boiled for 10 minutes. Subsequently, cool the solution to room temperature. Then centrifuge (5427 R centrifuge, Eppendorf, Germany) it at 6000 r/min for 5 minutes at 4°C. Collect the supernatant and freeze-dry (LyoQuest-55, Telstar, Germany) for 24 h. Use BCA kit
(Solarbio) to measure protein concentrations in a microplate and read at 562nm on a NanoDrop OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific). Dissolve the samples in 21μL MilliQ water containing 0.1% (v/v) formic acid for LC-MS/MS analysis.

**Liquid chromatography (LC)-electrospray ionization (ESI) tandem MS (MS/MS) analysis**

Each fraction was injected for nano LC-MS/MS analysis. The peptide mixture was loaded onto a reverse phase trap column (Thermo Scientific Acclaim PepMap100, 100 μm × 2 cm, nanoViper C18) connected to the C18-reverse phase analytical column (Thermo Scientific Easy Column, 10cm, ID75μm, 3μm, C18-A2) in buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (84% acetonitrile and 0.1% formic acid) at a flow rate of 300nL/min controlled by IntelliFlow technology. A two-hour gradient procedure was used as follows: 0 - 55% buffer B for 110 min, 55%- 100% buffer B for 5 min, 100% buffer B for 5 min. Then, use Q Exactive mass spectrometer (Thermo Scientific) for LC-MS/MS analysis. The mass spectrometer was operated in positive ion mode. The scanning range of precursor ion is 300-1800m/z. Automatic gain control (AGC) target was set to 1e6, and maximum inject time to 50ms. Dynamic exclusion duration was 60 s. Survey scans were acquired at a resolution of 70,000 at m/z 200 and resolution for HCD spectra was set to 17,500 at m/z 200, and isolation window was 2 m/z. Normalized Collision Energy was 30eV, Underll was 0.1%.

**Label free analysis**

The MS data were analyzed using Max Quant (version1.5.3.17, Max Planck Institute of Biochemistry, Germany). The MS data were searched against the uniport-Bos-taurus fasta. The following are relevant parameters: enzyme, trypsin/papain/pepsin; max missed cleavages, 2; fixed modification, carbamidomethyl; variable modification, oxidation; main search, 6 ppm; first search, 20 ppm; MS/MS tolerance, 20 ppm; database pattern, reverse; include contaminants, true; peptide FDR ≤0.01; protein FDR≤0.01; peptides used for protein quantification, razor and unique peptides; time window, 2 min; protein quantification, LFQ/iBAQ; min ratio count,1.

**Bioinformatics analysis**

Functional annotation and Network analysis were performed using STRING (http://string-db.org/) and Cytoscape platform version 3.8.2 (https://cytoscape.org) based on bos-taurus genes. In particular, the two plugins of Cytoscape, namely ClueGo (version 2.5.7) and CluPedia (version 1.5.7) were used to integrate the Gene Ontology (GO) categories (Biological Process(BP), Molecular Function (MF), Cellular Component (CC)), Reactome Pathways, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Wiki Pathways annotation⁹. The κ score level was set at ≥0.4 while minimum and maximum levels were set at 3 and 8, respectively.

**Results**

**Comparative evaluation of protein extraction efficiency in cow placenta hydrolyzed by three proteases**
The number of proteins identified from cow placenta with different digestion protocols differ significantly, 426, 115 and 57 in TRY, PEP and PAP, respectively (Table 1). Comparison of digestion efficiencies of three proteases showed that TRY produced significantly higher number of protein identifications than PEP and PAP. TRY extracted 3.7 times (t-test, p = 0.00132) and 7.5 times (t-test, p = 0.00015) more of proteins than PEP and PAP. Meanwhile PEP extracted 2 times more of proteins than PAP (t-test, p = 0.00678). Comparison of the common proteins among biological replicates of TRY and PEP revealed an overlap of 72–82%, while that of PAP ranged 53–77% (Fig. 1). TRY and PEP had higher reproducibility of acquisitions than PAP, with coefficient of variation (CV) from 2 to 4% for proteins and 8–10% for peptides, respectively, lower than 15% of proteins and 20% of peptides in PAP (Table 1). TRY, PEP and PAP extraction resulted in 541, 134 and 86 quantifiable proteins (proteins with LFQ intensity > 0) from cow placenta, respectively. Comparison of the quantifiable proteins of cow placenta among three proteases, common proteins were 80 (12.3%), 22 (3.4%) and 27 (4.1%) in TRY vs PEP, PEP vs PAP and PAP vs TRY, respectively. There were 449 (69.2%), 49 (7.6%) and 52 (8%) unique proteins in TRY, PEP and PAP, respectively (Fig. S1). Common proteins only constitute the minority of total quantifiable proteins, while unique proteins of TRY are the largest proportion.

Table 1

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<th>Sample name</th>
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<td>PAP</td>
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Note: The table lists numbers of identified peptides and proteins in each biological replicate, together with the average number of identification (Mean ± SD) and coefficient of variation (CV%).

Analysis of cow placenta quantifiable proteins whit three proteases

The quantifiable proteins of cow placenta whit trypsin, pepsin and papain were further analyzed in terms of distribution of proteins’ molecular weight (Mw), sequence coverage, peptides length and unique peptides ratio. Mw distribution is important in evaluating proteins size. In this study, Mw of quantifiable proteins extracted with three proteases was relative wide, and showed almost no difference. Mw results demonstrated that 71%, 76% and 67% proteins in TRY, PEP and PAP, respectively, were lower than 70kDa. Meanwhile, approximately 20% of the quantifiable proteins exceeded 100kDa (Fig. 2A). Sequence coverage determined the overall accuracy of detected proteins, while that was relatively high in the present study. There were 46%, 63% and 42% proteins with more than 10% sequence coverage distributions of quantifiable proteins (Fig. 2B). Peptides length revealed the characteristic of proteases. In this study, 92%, 76% and 88% detected peptides were lower than 20 in TRY, PEP and PAP, respectively (Fig.
2C). Additionally, each group consists its unique set of peptides endowing it with specific properties. Protein detection reliability tended to improve with the number of unique peptides in a protein group\(^\text{10}\). In the present study, the distribution curve of the number of unique peptides gradually increased, which means the number of both unique peptides and reliable proteins was relatively large (Fig. 2D).

**GO and KEGG analysis of cow placenta quantifiable proteins with three proteases**

GO analysis of cow placenta quantifiable proteins with three proteases showed highly similar distribution in biological progress, cellular component and molecular function (Fig. S2). The highest percentage are metabolic progress and biological regulation, followed by cellular component organization, response to stimulus and developmental process in biological progress. The quantifiable proteins mainly distributed in membrane, nucleus, protein-containing complex, cytosol and cytoskeleton in cellular component. Molecular function-based analysis showed that a majority of the proteins were involved in processes such as protein binding, ion binding, hydrolase activity, nucleotide binding, structural molecule activity and nucleic acid binding.

KEGG pathway analysis was carried out to understand the biological functions and the specific pathways related to cow placenta. Top 10 KEGG pathways in TRY, PEP and PAP are presented in Fig. S3. Focal adhesion, PI3K-Akt signaling pathway, Human papillomavirus infection and ECM-receptor interaction are common pathway in RY, PEP and PAP.

**Identification of differentially expressed proteins (DEPs)**

The DEPs were defined based on a 2.0-fold change threshold (with a fold change > 2.0 or < 0.50, p < 0.05) (Fig. 3) or specifically expressed (Table 2) in comparisons between groups according to mass spectrum data. PAP vs TRY detected 432 DEPs, including 34 up-regulated and 398 down-regulated (Fig. 3A, Table 2); PEP vs TRY detected 421 DEPs, including 56 up-regulated and 365 down-regulated (Fig. 3B, Table 2). PEP vs PAP detected 136 DEPs, including 100 up-regulated and down-regulated (Fig. 3C, Table 2).

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**Table 2**

Specially expressed protein in each group

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</tr>
<tr>
<td>Pepsin group vs Papain group</td>
<td>96</td>
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**Note:** "Presence" refers to proteins which consistent presence in the first group and absence in the other group; "Absence" refers to proteins which consistent presence in the second group and absence of the other group.
Cluster analysis of DEPs

The hierarchical clustering algorithm (Hierarchical Cluster) is used to perform cluster analysis on each group of DEPs, and the data is displayed as a heat map (Heatmap). Figure S4 shows that the DEPs screened by the standard of 2.0-fold change threshold (with a fold change > 2.0 or < 0.50, p < 0.05) can effectively separate the comparison groups, showing that they screened differentially expressed proteins can represent the difference between the two groups.

GO and KEGG enrichment analysis of the DEPs

Perform GO enrichment analysis on the up-regulated proteins and down-regulated proteins of each comparison group to compare the difference in bioinformatics of dairy cow placenta with different proteases. GO enrichment results show differences in biological information caused by proteases. The statistically significant (p < 0.05) network analysis performed using Cytoscape is shown in Fig. 4. DEPs enzymatic hydrolysis by papain enriched in collagen trimer, extracellular region part, extracellular exosome, chromaffin granule membrane and protein heterodimerization (Fig. 4A-B). DEPs enzymatic hydrolysis by trypsin enriched in blood microparticle, membrane region, complex of collagen trimer, extracellular organelle, extracellular matrix, extracellular matrix component, cell surface, viral nucleocapsid, regulation of locomotion, maintenance of location, syncytium formation and anatomical structure formation involved in morphogenesis (Fig. 4C-D). DEPs enzymatic hydrolysis by pepsin enriched in I band, immunological synapse, sarcomere, costamere, contractile fiber, structural molecule activity conferring elasticity, extracellular matrix binding, coagulation, structural constituent of muscle, NAD metabolic process, extracellular matrix structural constitution, collagen metabolic process, platelet activation, regulation plasma lipoprotein particle levels and cell death response oxidative stress (Fig. 4E-F).

In order to compare the difference in bioinformatics of dairy cow placenta with different proteases, KEGG enrichment analysis was performed on the up-regulated protein and down-regulated protein of each comparison group. Protein digestion and absorption Glycolysis / Gluconeogenesis Hypertrophic cardiomyopathy (HCM) Dilated cardiomyopathy (DCM) Focal adhesion Adherens junction Tight junction Leukocyte transendothelial migration Regulation of actin cytoskeleton PI3K-Akt signaling pathway Focal adhesion ECM-receptor interaction Regulation of actin cytoskeleton Amoebiasis are common enrichment pathway in each group (Fig. 5). The unique enrichment pathways of DEPs produced by papain are Relaxin signaling pathway and AGE-RAGE signaling pathway in diabetic complications (Figure. 5A-B). The unique enrichment pathways of DEPs produced by pepsin are Lysosome, Platelet activation, Cardiac muscle contraction, Bacterial invasion of epithelial cells, and Small cell lung cancer (Figure. 5C-D). The unique enrichment pathway of DEPs produced by trypsin is Arginine and proline metabolism, Olfactory transduction, Proteasome, Protein processing in endoplasmic reticulum, Pyruvate metabolism, Arrhythmogenic right ventricular cardiomyopathy (ARVC) (Figure. 5E-F).
Discussions

Proteomics has been widely applied to study the placenta related disease of human, but there are few studies related to placenta in dairy cow. Trypsin and pepsin are widely used in protein extraction and digestion in proteomics research\textsuperscript{11–14}, while papain is mostly used in the extraction of natural biologically active peptides\textsuperscript{15,16}.

In this study we carried out a comparative proteomic analysis of dairy cow placenta enzymatic hydrolysis by three proteases using a label-free MS approach. The main aim of this study was to give an objective, critical assessment on the performance of three proteases for digestion in proteomics of cow placenta. This study was based on the optimal enzymatic hydrolysis conditions of cow placenta with trypsin, pepsin and papain\textsuperscript{8}.

Comparison of the overall digestion efficiency between three proteases showed that trypsin was superior to pepsin and papain in number of protein identifications for cow placenta. We presume that trypsin superior efficiency is due to improved protein solubility and proteolytic efficiency of trypsin. Trypsin has the advantage of high specificity which cleaves exclusively at arginine (R) and lysine (K) residue\textsuperscript{17}, optimal pH closes to neutral\textsuperscript{18}, meanwhile the pH of amniotic fluid closes to 7.0. Therefore, we assumed that placental proteins are soluble in a neutral environment, which may be the primary reason of why trypsin enzyme solution efficiency is better than pepsin and papain. Jiao’s study reported that a total of 788 proteins were identified in placenta of healthy pregnant mice by label free proteomic with trypsin digestion\textsuperscript{19}. Wawrzykowski reported 886 proteins were identified in cow placenta by 2D separation with trypsin digestion\textsuperscript{20}. Compared with the above study, the amount of protein extracted by trypsin in this study is relatively small, which may be due to the direct enzymatic hydrolysis of placenta homogenate by trypsin, lack of detergents and denaturants such as RapiGest. In this study, there is room for improvement in protein digestion. However, the biological repetition rate of trypsin was more than 70\%, the molecular weight and sequence coverage were relatively wide, the peptides length conformed to the characteristics of trypsin digestion, and the unique peptides distribution curve increases gradually, all the above results notarized the reliability of proteome data.

Pepsin also has strong specificity, and the hydrolytic sites are aromatic and hydrophobic amino acids. A total of 110 proteins were identified by pepsin hydrolyzed mealworm in Bouklis’ research\textsuperscript{14}, which was closed to the result of this study, and the quality control data of pepsin was better than that of trypsin and papain, indicating that pepsin is reliable in proteomics. However, the number of identified proteins by pepsin is significantly less than that of trypsin, which may be caused by pH. Pepsin optimal pH rang 1.5 to 2.0\textsuperscript{21}, however some placental proteins have poor solubility in this acidic environment, which caused the low enzymatic hydrolysis efficiency of pepsin.

Papain were proteases that can release relatively larger quantity of bioactive peptides\textsuperscript{22}. Therefore, papain was mainly used for the extraction and digestion of natural active peptides in the current research. Papain hydrolyzed oat proteins had high ability to quench ABTS\%+ radicals and to chelate
ferrous ions while displaying the second strongest activity for ROO% radicals\textsuperscript{23}. Antioxidant activity of porcine liver hydrolysates using papain were relatively high\textsuperscript{24}. Papain can release high number of potential angiotensin converting enzyme (ACE)-inhibitory peptides from tilapia (Oreochromis spp.) processing co-products, frame and skin\textsuperscript{15}. In the above research, proteomic techniques were used analyze molecular characteristics of proteins. However, peptides generated with less than 5 amino acids (due to their high MWs and electrical charge) were not effectively detected by the MS/MS spectra\textsuperscript{25}. Thus, we speculate that most of the polypeptides hydrolysis by papain with less than 5 amino acids, which leads to a great number of potential bioactive peptides and a small number of protein identification, and the quality control data confirm this view, with a coefficient of variation of more than 15%, low protein repetition rate and molecular weight, narrow sequence coverage and peptide length distribution and few unique peptides.

Our results indicated that quantifiable proteins of cow placenta with trypsin, pepsin and papain were associated with almost same biological progress, molecular function and cellular component. The biological progress mainly included biological regulation and metabolic process, similar to the protein patterns in bovine placenta at early-mid pregnancy\textsuperscript{26}. The cellular component included protein-containing complex, membrane, cytosol nucleus and extracellular space. The main cellular component of identified proteins in retained and released bovine placenta were cytoplasm, nucleus and membrane\textsuperscript{20}. This result showed that the proteins that cause placental retention may be mainly distributed in cytoplasm. Classification of the quantifiable proteins, in accordance to molecular functions, revealed that the majority of proteins showed binding and catalytic activities, similar to the study of Ner-Kluza\textsuperscript{26}. In conclusion, the GO annotation of this study was almost similar to the current research. Further, the KEGG analysis indicated focal adhesion, PI3K-Akt signaling pathway, human papillomavirus infection and ECM-receptor interaction were common pathway in cow placenta with three proteases. Proteins important for adhesive processes were detected in retain bovine placenta and disturbances in the metabolism of extracellular matrix proteins what may lead to improper placental detachment. Our study confirmed focal adhesion and ECM-receptor interaction pathway were important in normal released placenta.

Different enzyme digestion sites leaded to different protein identification further induced the biological information differences. In order to explore these differences, up-regulated and down-regulated DEPs were analyzed in each comparison group. Predictively, the results were completely different.

Trypsin had been evidenced in previous studies can be used in proteomics such as pork, beef, chicken, fish, milk, and shrimp\textsuperscript{18,20,26−28}. Trypsin mainly enzymatically decomposes extracellular matrix, blood particles, and cell surface proteins. These proteins mainly perform positioning functions and participate in biological processes such as syncytium formation. Trypsin can be selected for the study of amino acid metabolism, proteasome and endoplasmic reticulum, dysosmia and arrhythmogenic right ventricular cardiomyopathy.

Pepsin mainly enzymatically degrades myofibrils, muscle fiber l-bands, actin ribs and muscle contraction fibers, has the function of activating elastic fibers and linking extracellular matrix, and is involved in blood
coagulation, muscle structure composition, NAD metabolism, and extracellular matrix composition. Biological processes such as collagen metabolism and cell apoptosis caused by oxidative stress. The above results indicate that pepsin can significant hydrolysis effect on muscle tissue in the placenta, and more biological information related to muscle tissue can be identified. When studying muscle tissue-related proteomics, pepsin digestion may be used to obtain more comprehensive biological information. Pepsin can be selected to study diseases such as lysosome, platelet aggregation and myocardial contraction, bacterial invasiveness and small cell lung cancer.

Papain has a strong ability to decompose extracellular matrix and collagen and can be used in Relaxin signaling pathway and AGE-RAGE signaling pathway in diabetic complications research according to GO and KEGG results

Conclusion

This study identified the differential proteins produced by the enzymatic hydrolysis of cow placenta by different proteases. The production of these differential proteins is related to the characteristics of protease cleavage. In particular, proteins related to protein binding and ion binding protein-containing complex and membrane metabolic process and biological regulation were detected in various enzymatic hydrolysis products. All in all, these findings explain the basic biological information of healthy dairy cow placenta and are helpful for the screening of specifically expressed proteins and biomarkers in dairy cow pregnancy diseases. At the same time, it can provide guidance on the selection of protease for specific tissues and specific directions for proteomics.

Declarations

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Data Availability Statement

Supplementary Table S1 provide proteins identification list; Supplementary Table S2 list peptides identified. The datasets generated and analyzed during the current study are available in the Mendeley Data repository, https://data.mendeley.com/datasets/sh58hkzfmd/1.

References

**Figures**
Figure 1

Protein overlap between replicates from cow placenta extracted by different proteases. Proteins obtained from cow placenta with trypsin (A), pepsin (B) and papain (C) were run in triplicate biological replicates (BR). Data were processed using the MaxQuant (version 1.5.3.17) with protein FDR 1%.
Figure 2

Qualitative and quantitative analysis of quantifiable proteins in cow placenta extracted with trypsin, pepsin and papain. Analysis was done based on the distribution of proteins according to: (A) molecular weight, (B) Sequence coverage, (C) Peptides length, (D) Unique peptides number.
Figure 3

**Volcano plot showing differentially abundant proteins between each group.** (A) papain group vs trypsin group; (B) pepsin group vs trypsin group; (C) pepsin group vs papain group. The X-axis is the negative log of the Fold-Change with 2 as the base, and the Y-axis is the negative log of the p-value with 10 as the base. Gray dots show no significant difference protein; red dots show significantly up-regulated proteins, and green dots show significantly down-regulated proteins.

Figure 4

**GO enrichment analysis of DEPs.** papain group vs trypsin group up-regulation protein (A); papain group vs pepsin group down-regulation protein (B); papain group vs trypsin group down-regulation protein (C); pepsin group vs trypsin group down-regulation protein (D); pepsin group vs trypsin group up-regulation protein (E); pepsin group vs papain group up-regulation protein (F). The node color indicates biologically similar reactions, and the size reflects the number of genes contributing to the pathway. If the reaction pathway shares 50% or more of the contributing genes, then they are connected by an edge. The representative nodes (based on FDR) are indicated by the colored texts (same below).
Figure 5

**KEGG enrichment analysis of DEPs.** Papain group vs trypsin group up-regulation protein (A); papain group vs pepsin group down-regulation protein (B); papain group vs trypsin group down-regulation protein (C); pepsin group vs trypsin group down-regulation protein (D); pepsin group vs trypsin group up-regulation protein (E); pepsin group vs papain group up-regulation protein (F). The shade of colors indicated the size of $P$ value.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigures.docx
- supplementarytableS1.xlsx
- supplementarytableS2.xlsx