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

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Research

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Abstract

Background

Novel findings in biochemical characteristics of placenta can bring new insight for further studies on the potential markers of physiological/pathological pregnancy or function of placenta. In this study, we compared the proteome of dairy cow placenta enzymatic hydrolysis by different proteases using a label-free mass spectrometry approach.

Results

In total, 541, 136 and 86 proteins were identified in trypsin group (TRY), pepsin group (PEP) and papain group (PAP), respectively. 432 differentially expressed proteins (DEPs) were identified between the proteomes of PAP and TRY. 421 DEPs were identified between PEP and TRY, while 136 DEPs were identified between PEP and PAP. Further analysis of the up-regulated DEPs and down-regulated DEPs of each comparison showed the proteins identified by papain are mostly derived from extracellular matrix and collagen and enriched in relaxin signaling pathway, advanced glycation end products-receptor of advanced glycation end products (AGE-RAGE) signaling pathway in diabetic complications. Moreover, pepsin digestion resulted in more muscle-related proteins, which 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).

Conclusions

In summary, these data provide insights into the selection of protease in dairy cow placenta proteomics.

Background

The placenta is an organ that connects the mother and the fetus to maintain a stable environment for the growth and development of fetus in mammalian. It regulates the growth and development of fetus by regulating the supply of nutrients, gases, hormones, etc. and exhibits essential substance exchange, hormone secretion and barrier effects[1]. The placenta alters the mother's endocrine system and immune system, forms a blood vessel link between the mother and embryo, which is key to maintaining the growth of fetus[2]. Abnormal placental function is one of the principal causes of fetal death. Proteomic technology is a common tool for studying placental abnormalities. It helps understand the alteration of placental protein during the disease process, clarify the pathogenesis and search for differentially expressed proteins related to the pathogenesis, serving as an effective tool for clinical diagnosis of the disease. Placental proteomics can also be used to discover mare peptides and related biomarkers[3].

Using placental proteomics, it has been shown that differential proteins in the placenta of human preeclampsia are closely related to mitochondrial function, indicating that mitochondrial dysfunction is a precursor of the epilepsy pathogenesis [4]. Moreover, it has been 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) through placental proteomics [5].

The digestion and extraction of proteins by protease is an important factor during the process of proteomics. Currently, trypsin is often used in placental proteomics for protein extraction. Trypsin is highly specific, only cutting arginine (R) and lysine (K) residues[6], so proteins lacking arginine and lysine residues cannot be digested by trypsin. In addition, when the trypsin cleavage site was located after the glycosylated asparagine, the attached carbohydrates may sterically prevent trypsin cleavage[7]. In order to overcome the limitations of trypsin, non-specific enzymes, such as papain and pronase, can be used to completely digest the protein. Trypsin is most effective in a neutral or slightly alkaline environment, but proteins with low solubility in this environment cannot be digested. Pepsin is of 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 conditions for enzymatic hydrolysis of cow placenta hydrolyzed by trypsin, pepsin and papain with high reducing activity and extraction rate has been established, and the label-free technology was used to analysis hydrolysis products. Qualitative and quantitative analysis of the protein and peptides obtained from cow placenta are used 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.

Methods

Placenta collection and preparation

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. 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 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[8]. 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 One^{C} 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, Underfill 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 annotation[9]. 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. Common proteins only constitute the minority of total quantifiable proteins, while unique proteins of TRY are the largest proportion.

Table 1
Protein and peptide identifications from cow placenta.

Sample name	Proteins						Peptides					
	BR 1	BR 2	BR 3	Mean	SD	CV (%)	BR 1	BR 2	BR 3	Mean	SD	CV (%)
TRY	401	443	430	425	18	4	2022	2489	2002	2171	225	10
PEP	117	111	113	114	2	2	993	939	816	916	74	8
PAP	64	59	44	56	8	15	99	98	62	86	17	20

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 with 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, but 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. 3A). 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. 3B). 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. 3C). 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[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. 3D).

GO and KEGG analysis of cow placenta quantifiable proteins whit 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. 4). The highest percentage is 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 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. 5. Focal adhesion, PI3K-Akt signaling pathway, Human papillomavirus infection and ECM-receptor interaction are common pathways in RY, PEP and PAP.

Identification of differentially expressed proteins

The DEPs were defined based on a threshold of 2.0-fold change (with a fold change > 2.0 or < 0.50, $p < 0.05$) (Fig. 3) or specially 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. 6A, Table 2); PEP vs TRY detected 421 DEPs, including 56 up-regulated and 365 down-regulated

(Fig. 6B, Table 2). PEP vs PAP detected 136 DEPs, including 100 up-regulated and down-regulated (Fig. 6C, Table 2).

Table 2
Specially expressed proteins in each comparisons group.

Comparisons	Consistent presence /absence expression profile	
	Presence	Absence
Papain group vs Trypsin group	31	391
Trypsin group vs Pepsin group	47	340
Pepsin group vs Papain group	96	34

" 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. 7 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 the screened differentially expressed proteins can represent the difference between the two groups.

GO and KEGG enrichment analysis of the DEPs

We performed GO enrichment analysis on the up-regulated proteins and down-regulated proteins of each comparison to understand the difference in bioinformatics of dairy cow placenta hydrolyzed with different proteases. GO enrichment results showed differences in biological information due to different proteases. The statistically significant ($p < 0.05$) network analysis performed using Cytoscape was shown in Fig. 6. DEPs enzymatic hydrolysis by papain enriched in collagen trimer, extracellular region part, extracellular exosome, chromaffin granule membrane and protein heterodimerization (Fig. 6A-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. 6C-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. 6E-F). In order to compare the difference in bioinformatics of dairy cow placenta hydrolyzed 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 trans-endothelial 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. 8). The unique enrichment pathways of DEPs produced by papain are Relaxin signaling pathway and AGE-RAGE signaling pathway in diabetic complications (Fig. 8A-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 (Fig. 8C-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, ARVC (Fig. 8E-F).

Discussions

Proteomics has been widely used in the study of placenta related disease in human, but were rarely used in studies related to placenta in dairy cow. Trypsin and pepsin are commonly used in protein extraction and digestion in proteomics research[11–14], while papain is mostly used in the extraction of natural biologically active peptides[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 investigate an objective, critical assessment on the performance of three proteases for hydrolysis in proteomics of cow placenta. This study was based on the optimal enzymatic hydrolysis conditions of cow placenta with trypsin, pepsin and papain[8].

Identification of cow placenta proteins hydrolysis by trypsin, pepsin and papain

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's 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[17], optimal pH closes to neutral[18], meanwhile the pH of amniotic fluid closes to 7.0. Therefore, we hypothesized that placental proteins are soluble in a neutral environment, which may be the primary reason of why trypsin hydrolysis 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[19]. Wawrzykowski reported 886 proteins were identified in cow placenta by 2D separation with trypsin digestion[20]. Compared with the above studies, the number of proteins extracted by trypsin in this study is relatively low, 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 possibility for improvement in enzymatic 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 proved the reliability of proteome data. Pepsin is also highly specific, and the hydrolytic sites are aromatic and hydrophobic amino acids. A total of 110 proteins were identified by pepsin hydrolyzed mealworm in Bouklis' research[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 ranged 1.5 to 2.0[21], however some placental proteins have poor solubility in this acidic environment, which caused the low enzymatic hydrolysis efficiency of pepsin. Papain was protease that can cleave relatively larger quantity of bioactive peptides[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 displayed the second strongest activity for ROO% radicals[23]. Antioxidant activity of porcine liver hydrolysates using papain were relatively high[24]. Papain can cleave high number of potential angiotensin converting enzyme (ACE)-inhibitory peptides from tilapia (*Oreochromis spp.*) processing co-products, frame and skin[15]. In the above researches, proteomic techniques were used to analyze molecular characteristics of proteins. However, peptides generated with less than 5 amino acids (due to their high MWs and electrical charge) can't be effectively detected by the MS/MS spectra[25]. Thus, we speculate that most of the polypeptide hydrolysis by papain were less than 5 amino acids, leading to a great number of potential bioactive peptides and a small number of protein identification. Moreover, the quality control data confirm this speculation, 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.

Bioinformation of normal cow placenta and the feature of trypsin, pepsin and papain

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[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[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[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 hydrolysis 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 delivered placenta. Different enzyme digestion sites leaded to different protein identification further induced the biological informatic 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 proved in previous studies can be used in proteomics such as pork, beef, chicken, fish, milk, and shrimp[18, 20, 26–28]. Trypsin mainly enzymatically decomposed extracellular matrix, blood particles, and cell surface proteins. These proteins mainly perform positioning functions and participate 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 I-bands, actin ribs and muscle contraction fibers, those have the function of activating elastic fibers and linking extracellular matrix, and are involved in blood coagulation, muscle structure composition, NAD metabolism, and extracellular matrix composition. The above results indicate that pepsin can effectively hydrolyze the 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 differences in proteins produced by the enzymatic hydrolysis of cow placenta by different proteases. The production of these differential proteins is related to the enzymatic 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. Furthermore, these findings help explain the biological information of healthy dairy cow placenta and are helpful for the screening of specifically expressed proteins and biomarkers in dairy cow pregnancy diseases. In the meantime, it provides guidance on the selection of protease for specific tissues and specific directions for proteomics.

Abbreviations

TRY: Trypsin group; PEP: Pepsin group; PAP: papain group; DEPs: Differentially expressed proteins; AGE-RAGE: Advanced glycation end products-receptor of advanced glycation end products; ARVC: arrhythmogenic right ventricular cardiomyopathy; AGT: Angiotensinogen; F2: Prothrombin; R: Arginine; K: lysine; LC-MS/MS: Liquid chromatography electrospray ionization tandem mass spectrometer; AGC: Automatic gain control; BP: Biological process: MF: Molecular function; CC: Cellular component (CC);

KEGG: Kyoto encyclopedia of genes and genomes; MW: molecular weight; GO: Gene Ontology; HCM: Hypertrophic cardiomyopathy; DCM: Dilated cardiomyopathy.

Declarations

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Authors' contributions

L. H. Shen and S. Z. Cao: conceived the study and its animal design. Y. Zhang, L. C. You participated to the animal design and sample collection; Y. K. Zhu, Y. Shen, S. K. Lv: performed statistics, analyzed the data and interpreted the results; Y. Zhang: Writing-original draft. S. M. Yu, J. L. Deng, G. N. Peng and Z. C. Zuo: supervision and validation X. P. Ma Z. J. Zhong, Z.H. Ren and X. L. Zong: writing-review and editing. All authors helped to draft the manuscript and read and approved the final version.

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Availability of data and materials

Mean values of all data generated or analyzed during this study, mass spectrometry parameters and detailed identification of proteins are included in this published article and its additional information files. Individual data are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Cows were cared in compliance whit Care and Use of Laboratory Animals of China, and all procedures were approved by the Animal Care and Use Committee of Sichuan Agricultural University.

Consent for publication

Not applicable.

Competing interests

The authors declare that we have no competing interests

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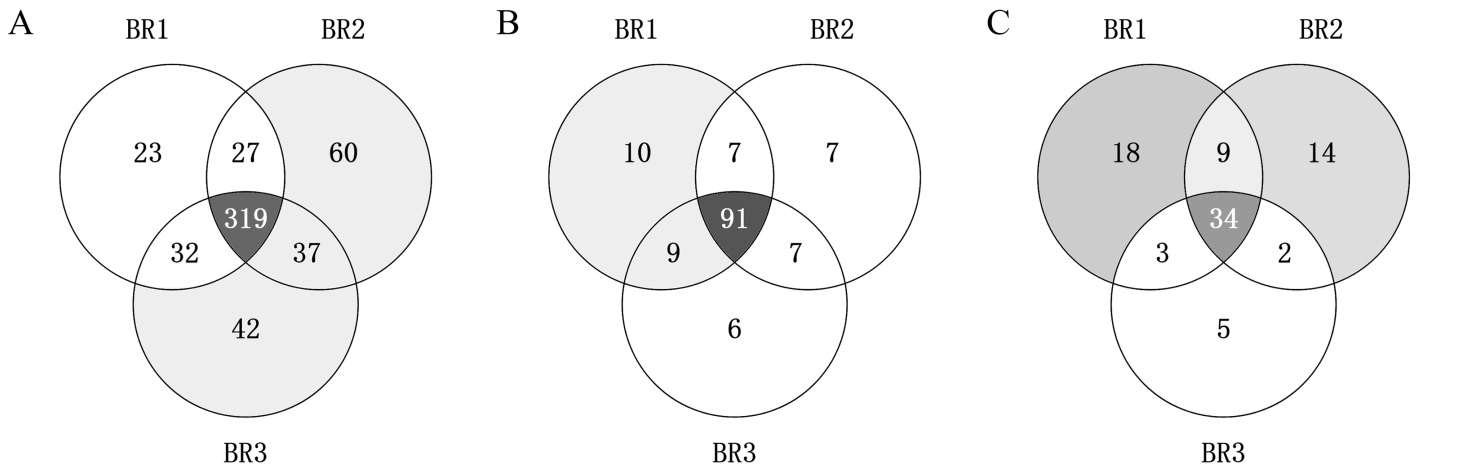
Figures



Common proteins (%)			Common proteins (%)			Common proteins (%)		
BR1	BR2	BR3	BR1	BR2	BR3	BR1	BR2	BR3
80	72	74	78	82	81	53	58	77

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%.

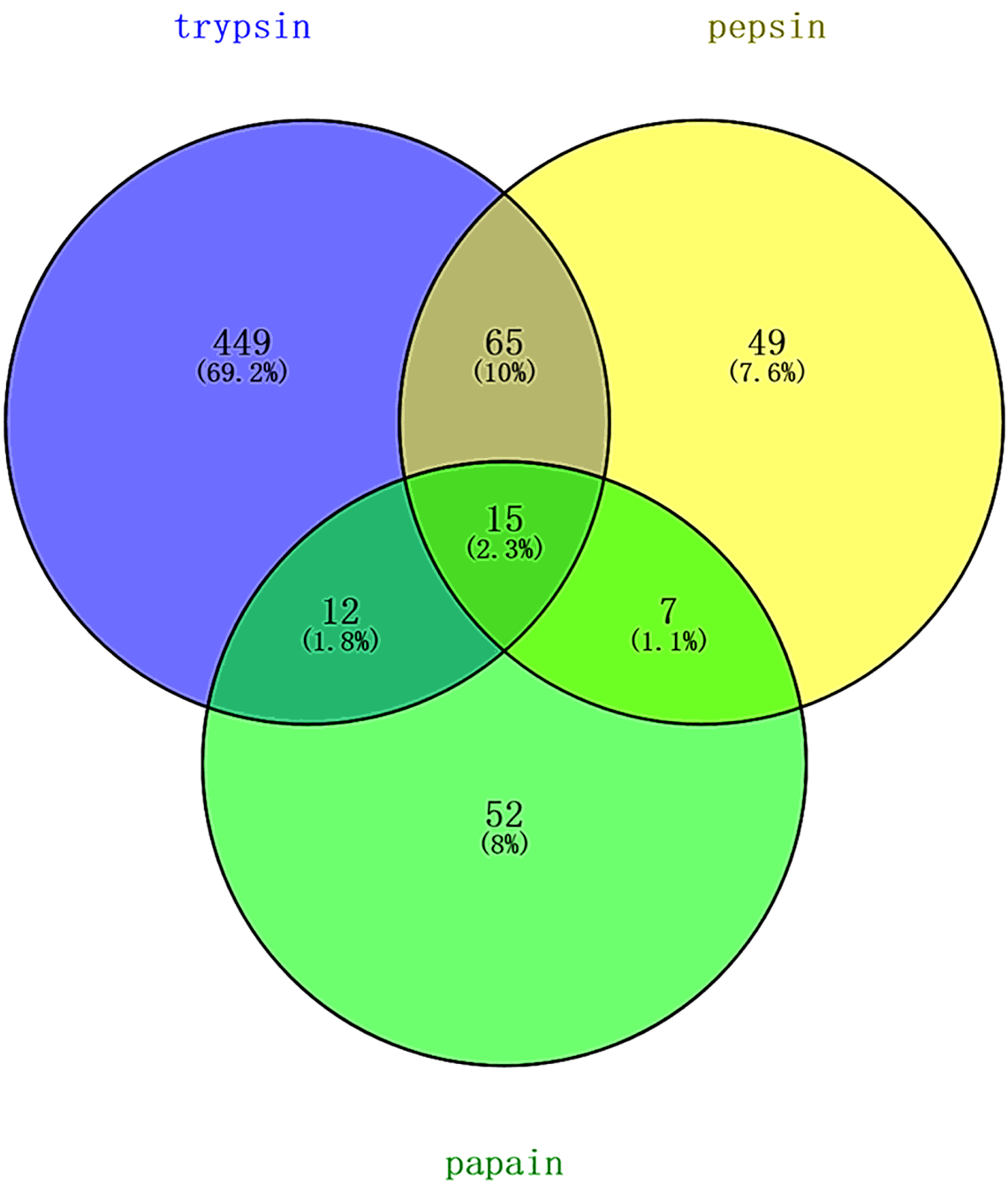


Figure 2

Identified common and unique quantifiable proteins between TRY, PEP and PAP. For each comparison, total number of the quantified proteins per protease as well as common and unique proteins have been presented as number and percentage.

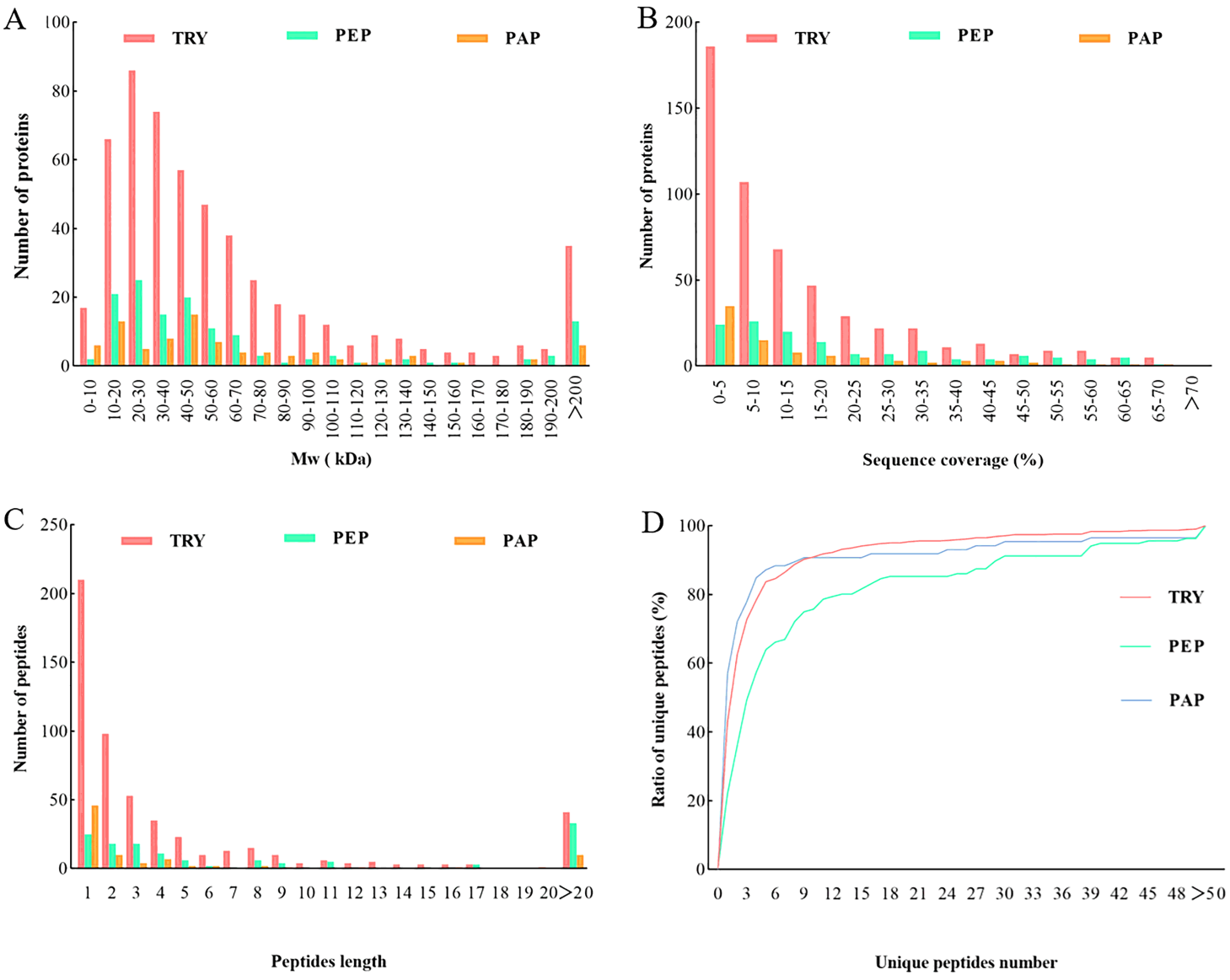


Figure 3

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.

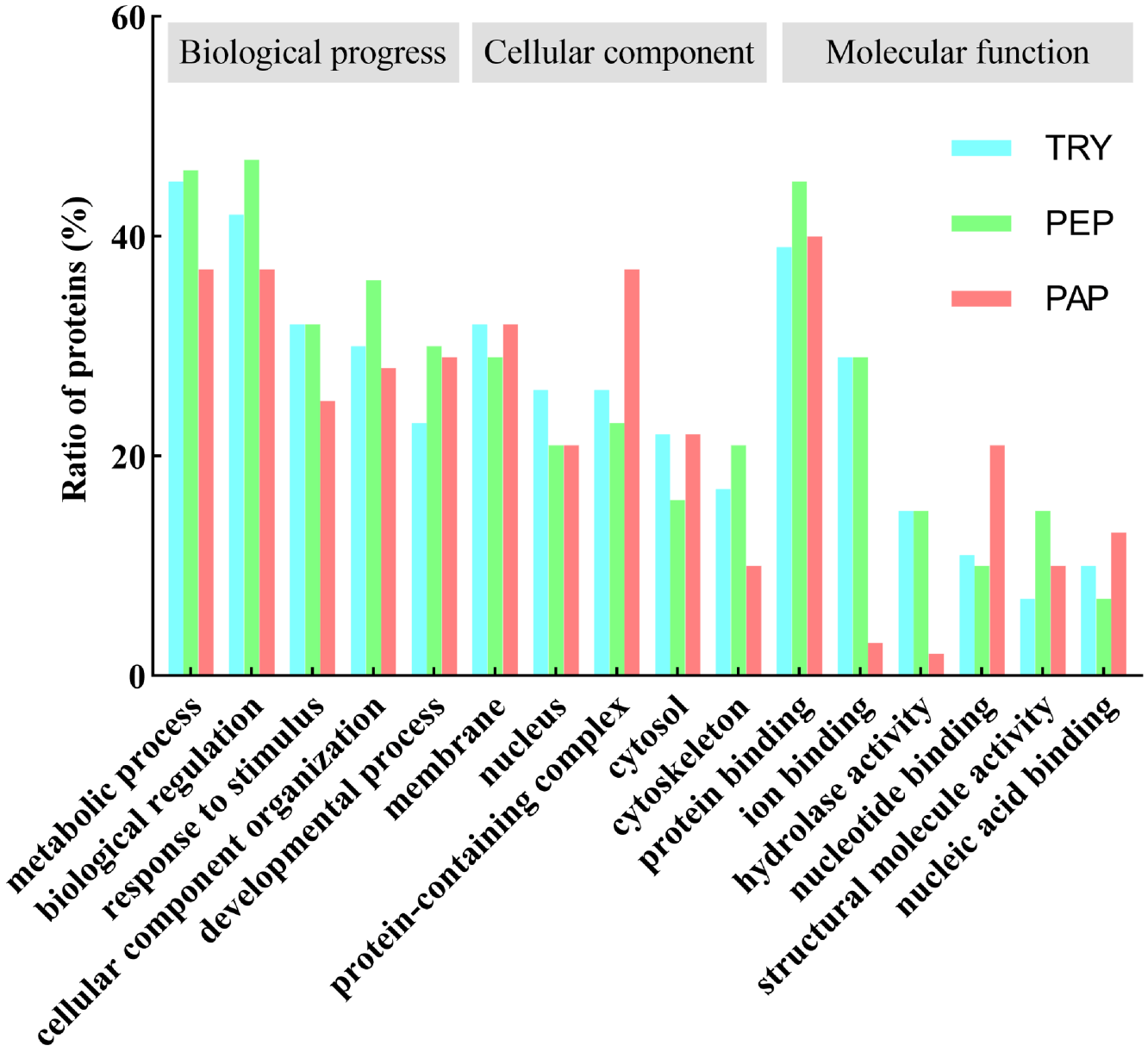


Figure 4

Gene ontology (GO) analysis of quantifiable proteins. Functional assignments of proteins' corresponding associated biological processes, molecular function and cellular components are shown. The number of DEPs that could be assigned to the different categories was indicated. Blue column indicates TRY; green column indicates PEP; red column indicates PAP.

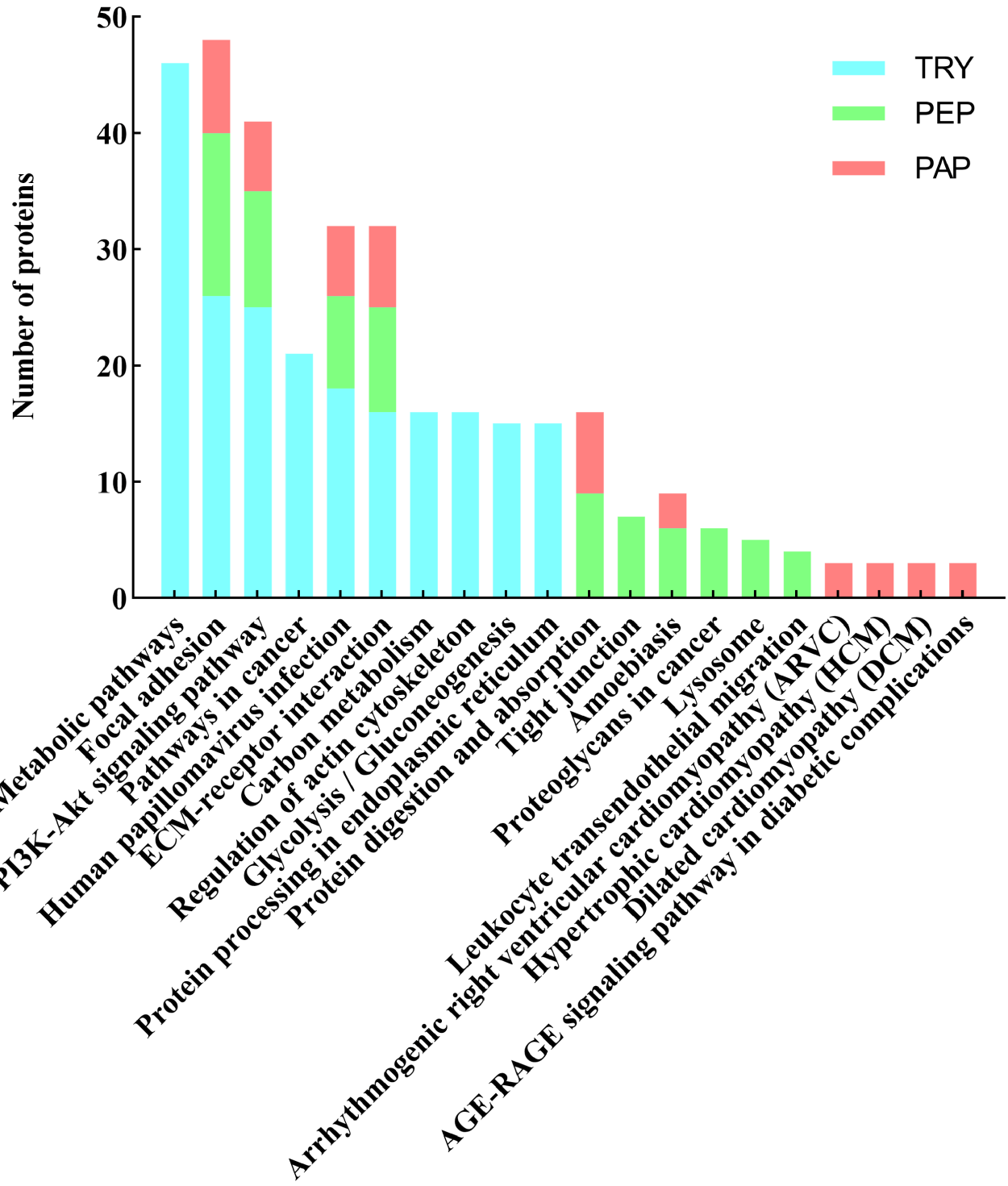


Figure 5

KEGG analysis of quantifiable proteins (Top10). Blue column indicates TRY; green column indicates PEP; red column indicates PAP. Under the x-axis are KEGG terms. Above each term show the number of quantifiable proteins.

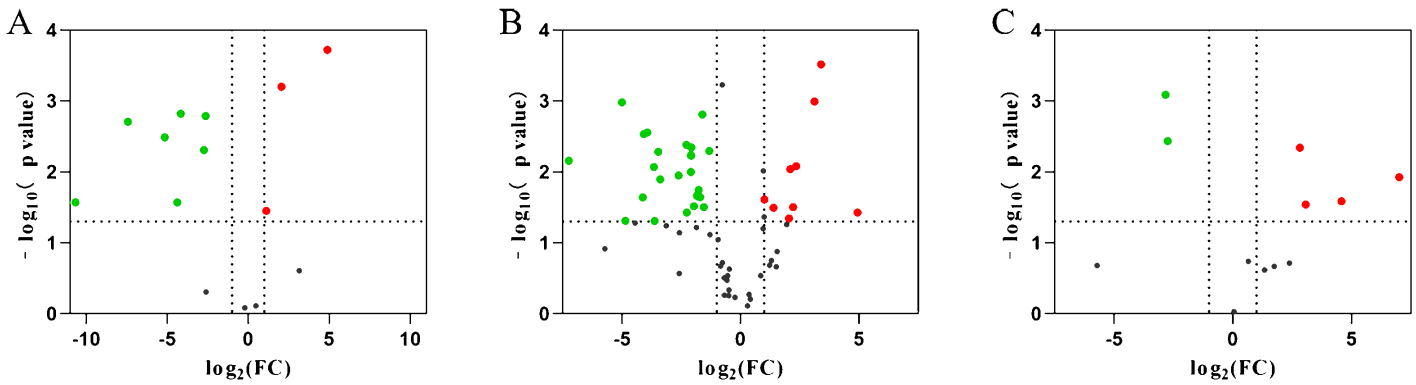


Figure 6

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.

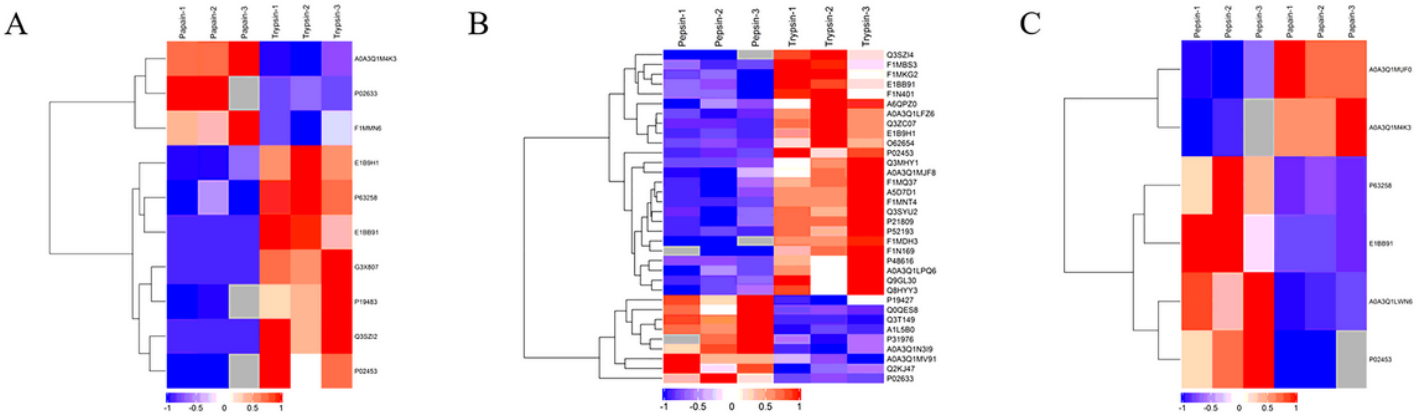


Figure 7

Cluster analysis of DEPs. Trypsin group vs papain group (A); trypsin group vs pepsin group (B); papain group vs pepsin group (C). The hierarchical clustering results are represented by tree heat maps, in which each row represents a protein (that is, the ordinate is the protein with significant difference), and each column represents a group of samples (Abscissa is the sample information). The logarithmic values (Log2Expression) of the protein expression of significant difference in different samples are shown in the heat map in different colors, in which red represents significantly up-regulated protein, blue represents significant down-regulated protein and gray part represents no protein quantitative information.

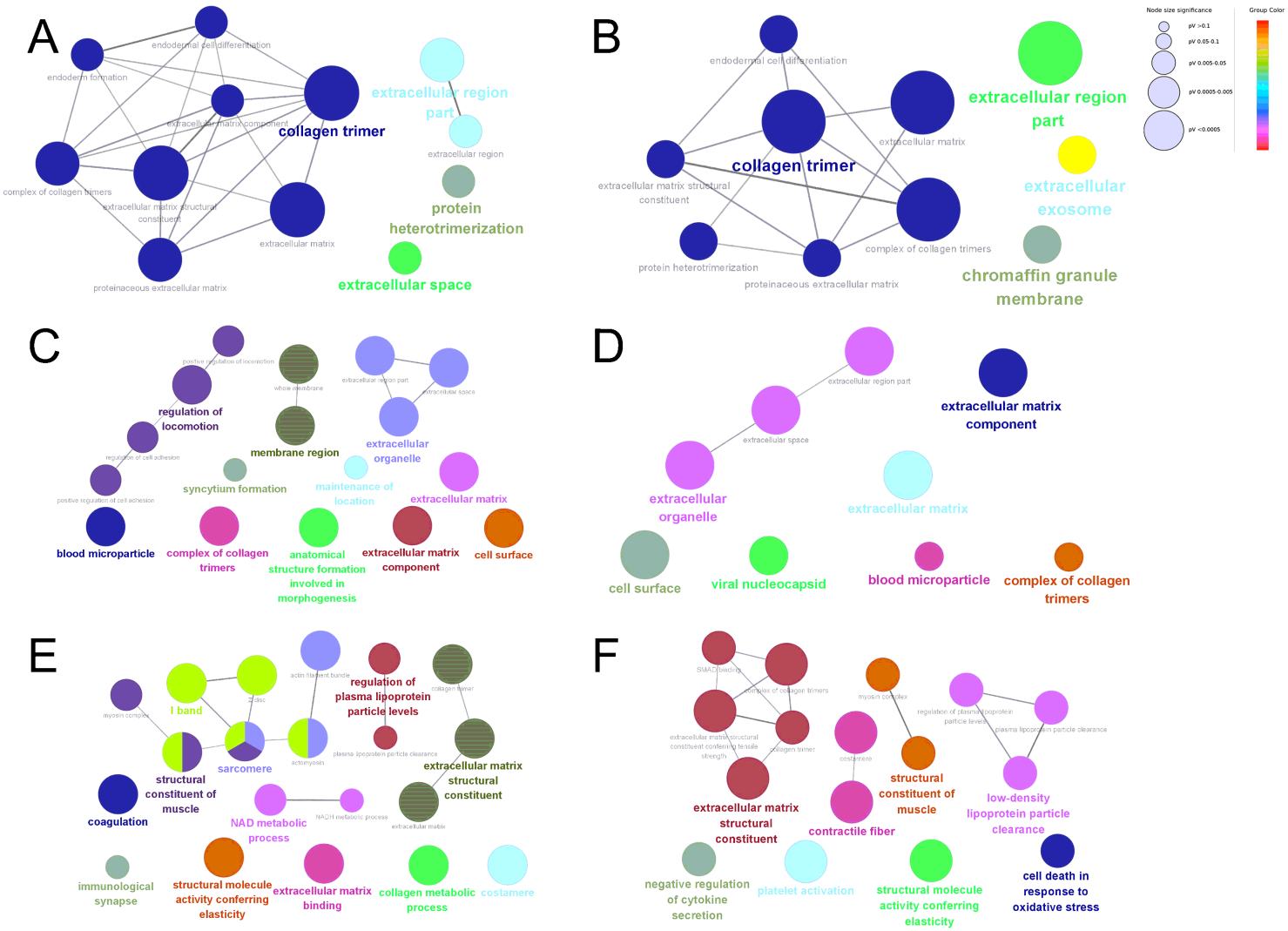


Figure 8

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).

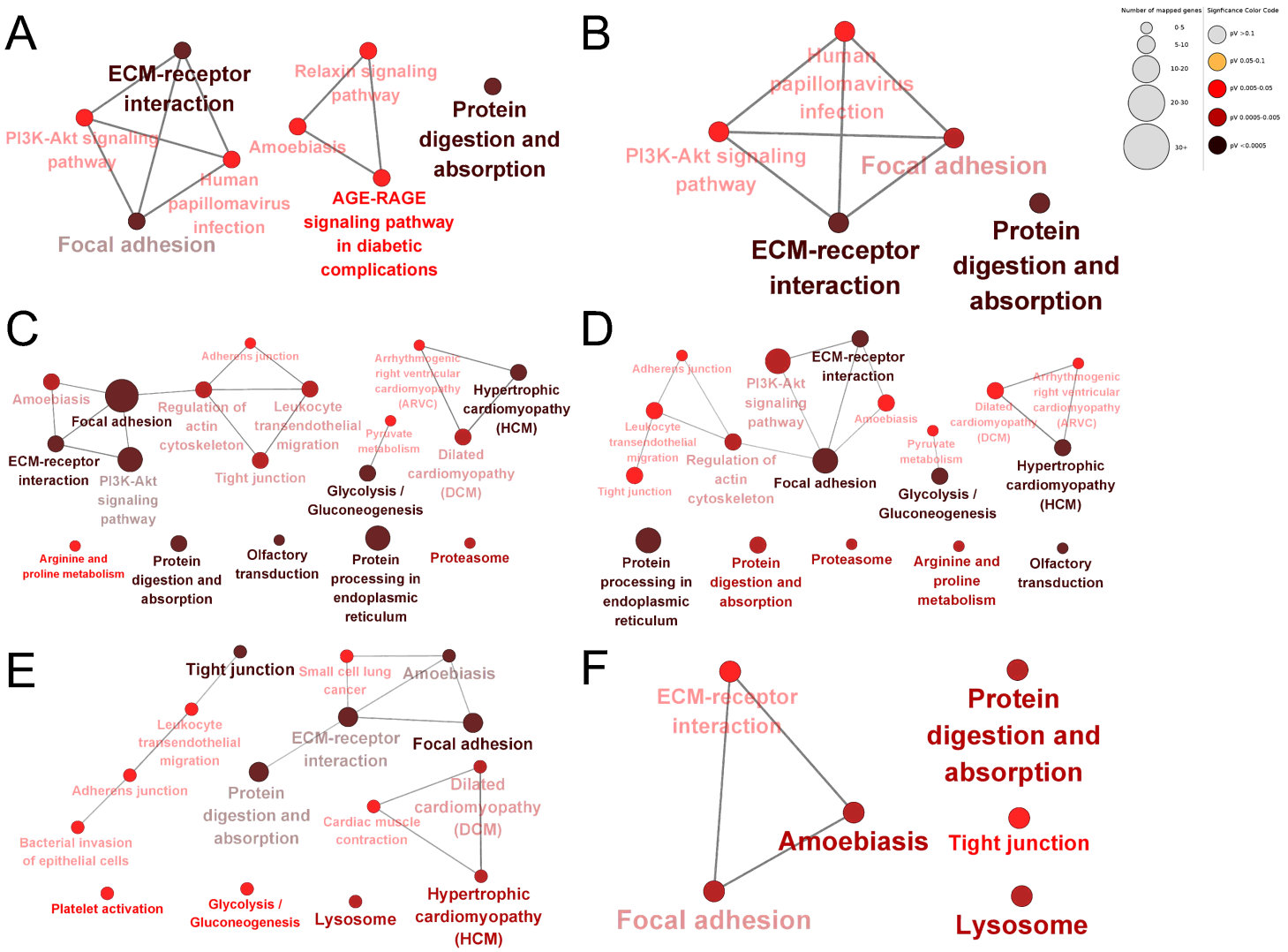


Figure 9

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.

- Additionalfile1.xlsx
- Additionalfile2.xlsx