Proteomic analysis of *Corylus heterophylla fisch* in Changbai Mountain by shotgun approach

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Abstract: Total proteins of Changbai Mountain hazelnut were extracted and then enzymolysed in solution. The mixture of peptides was separated on chromatograph and identified by shotgun proteomics approach. The identified proteins were analyzed by bioinformatics, 303 proteins were identified, of which 237 proteins (78.2%) were mainly distributing in a range of 10-70 kDa and 85 proteins (28.1%) were around pI 5-6. Based on the biological process cellular component and molecular function, these proteins were classified by Gene Ontology, the results showed that 183 proteins (73.8%) had catalytic activity (e.g. oxidoreductase, kinase, peptidase, etc.), and 170 proteins (68.5%) had binding activity. The protein profile provided a valuable basis for further research of hazelnut proteins and opened up new research avenues related to the function of these proteins.

Keywords: Changbai Mountain hazelnut; protein expression profile; shotgun proteomics.

INTRODUCTION

The increasing global economic problems and consumer demands originated from health concerns, religious limitations and rising trend of vegetarianism have recently arisen an interest in the usage of functional plant based proteins as alternative to animal proteins in the food industry (Levent *et al*, 2014).

Hazel nuts are consumed all over the world in dairy, bakery, candy and chocolate products. Besides being highly nutritious, hazel nuts have other properties that might be useful in the development of products other than foods, for example, cosmetics and pharmaceuticals (Teresa *et al*, 2010), they have an important role in human nutrition and health due to their protein, oil, vitamin and mineral content. Hazel nuts are rich in both monounsaturated and polyunsaturated fatty acids, as well as in vitamin E, which is worth to be studied (Ozdemir *et al*, 2001).

The concept of proteome was first proposed by the Australian scholar Wilkins (Wilkins *et al*, 1996). In recent years, proteomics method has developed rapidly and been applied in a number of food investigations. As an important tool for proteomics analysis, shotgun proteomics has been widely used to construct differential protein expression profile, it could identify hundreds of proteins in single run from little sample without complicated preparation. (Zhong-Quan *et al*, 2012, Chiara *et al*, 2013, Hasani-Ranjbar, *et al*, 2010, McDonald *et al*, 2002, Momtaz, *et al*, 2010). The method has been proved to be a successful approach in protein expression construction. But in recent years, proteomic technique applying in hazelnuts focus on allergen determination, now at least five proteins have been reported as potential food hazelnut allergens (Yu-Wei *et al*, 2011). The number of protein which have been identified in NCBI is not as much as other plant proteins. In this study, hazelnut proteins were characterized by combined proteomic methodologies.

MATERIALS AND METHODS

Materials

Hazelnut protein isolate (content of protein: 85.20%, prepared by the method of alkaline dissolving and acid precipitating, Protein content were determined by the Kjeldahl method (6.25×N)).

All reagents were analytical pure or higher grade. Chemicals, acetone, acetonitrile (ACN), formic acid (FA), Tris-hydroxymethyl aminomethaneand (Tris), caffeine, polypeptide MRFA, polymers Ultramark1621, Trifluoroacetic acid (TFA) were from Sigma (USA). TPCK-Trypsin, dithiothreitol (DTT), iodoacetamide (IAA) were from Promega; RP-C18 were from CTI company (Column: 0.15mm×150mm); Zorbax 300SB-C18 Trap column were from Agilent Technologies; LTQ were from Thermo Finnigan; filter tube were from Millipore.

Methods

Trypsin hydrolysis

Briefly, 30μL STD buffer were added to treat 30μg protein samples at 100°C and cooled to room temperature, then added 200μL UA buffer (8M Urea, 150mM Tris-HCl, pH8.5) and mixed for centrifugation using 30kDa

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ultra filtration tube and the filtrate was discarded. Subsequently, alkylated with 100μL iodoacetamide (50mM IAA in UA) for 30 min in the dark at room temperature after oscillating 1 min and centrifuged, then a volume of 10μL UA buffer was added and centrifuged, duplicated twice. Added 25mM NH_4HCO_3 and centrifuged, duplicated twice. At last, the samples were digested in digestion buffer with 40μL trypsin for 20h at 37°C. The supernatant containing the peptide mixtures was collected and then suspended with 0.1% methanoic acid before capillary high performance liquid chromatography (Michelle *et al*, 2013).

Capillary high performance liquid chromatography

All peptide fragments were separated by capillary high performance liquid chromatography. The flow rate was split from 200μL/min to 5μL/min using a flow splitter. Eluents: (A) 0.1% methanoic acid in water; and (B): 0.1% formic acid in 84% acetonitrile.

Peptides were separated on capillary column, C18 PepMap, 150 mm in length, 0.15mm ID and eluted using a 4%-50% linear gradient of solvent B in 0~30 min, 50%-100% B in 30min~34min, 100% B in 30min~34min.

MS analysis

The experiments were performed with LTQ Orbitrap XL, which could use Nan spray for peptide detection as following: after 20 fragments acquired during each full scan, original files were analyzed against the protein database by using Maxquant software. Orbitrap was used to quantitative instead of LTQ, the linear range can above 100000,which coule completely quantitative different size of molecular. Multiple peptide identifications were generally returned by Maxquant for each MS spectrum and for each parent-ion change state. The parameter used in our searching was as follows: Fixed modifications: Carbamidomethyl (C); Max Missed Cleavages: 2; Protein FDR <=1%, Peptide FDR <=1%; Min. Peptide Length >=7 aa; Variable modifications: Oxidation (M); Acetyl (Protein N-term); Protein FDR <=1%, Peptide FDR <=1%。Two or more peptides identified in this way are usually sufficient to unambiguously identify a protein. Moreover, BLAST analysis was necessary if peptides were matched to multiple proteins.

Gene Ontology (GO) annotation

To identify protein, sequences searches were performed with Inter Pro scan software (http://www.ebi.ac.uk/Tools/pfa/iprscan/) (Zdobnov *et al.*, 2001). The results of matched terms were outputted in RAW format. Then based on Web Gene Ontology Annotation Plot (WEGO), the compiled RAW outputs were subjected to GO categories (Ana *et al*, 2005, Ye *et al*, 2006).

KEGG pathway

KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information.

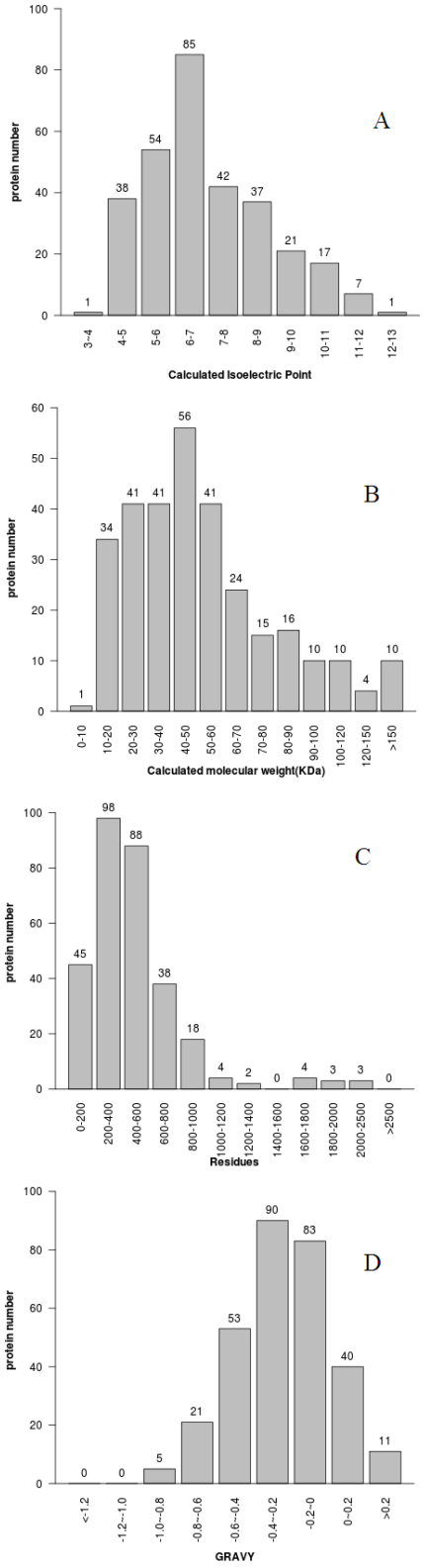

Fig. 1: A) pI distribution. B) MW distribution. C) Residues number distribution. D) GRAVY value distribution.

Table 1: The list of hazel nut proteins

Accession	KEGG_PATHWAY	Accession	KEGG_PATHWAY
11133373	ko00020: Citrate cycle (TCA cycle);	11133775	Ko 04141: Protein processing in endoplasmic reticulum
1168410	ko00010: Glycolysis / Gluconeogenesis;	1170878	Ko 00620: Pyruvate metabolism;
118574756		122045024	Ko 05034: Alcoholism;
12585330	ko00010: Glycolysis / Gluconeogenesis;	12643762	Ko 00250: Alanine, aspartate and glutamate metabolism;
126773	ko00620: Pyruvate metabolism;	1346672	Ko 00230: Purine metabolism;
1351202	ko04145: Phagosome;	2499489	Ko 00051: Fructose and mannose metabolism
2501187		2811030	Ko 00250: Alanine, aspartate and glutamate metabolism
3121727	ko00010: Glycolysis / Gluconeogenesis;	34922495	Ko 00071: Fatty acid metabolism;
351721013	ko00071: Fatty acid metabolism;	351721638	Ko 00010: Glycolysis / Gluconeogenesis;
351722759	ko04142: Lysosome;	351723511	Ko 00520: Amino sugar and nucleotide sugar metabolism
351723803	ko03010: Ribosome	351724053	Ko 00380: Tryptophan metabolism;
351724617	ko03010: Ribosome	351724625	
351725109	ko04141: Protein processing in endoplasmic reticulum	351725641	Ko 00061: Fatty acid biosynthesis;
351726138	ko03010: Ribosome	351727206	Ko 00010: Glycolysis / Gluconeogenesis;
351727653	ko03010: Ribosome	351727993	
358248130		358248740	Ko 03010: Ribosome
358248782	ko04360: Axon guidance;	358248794	Ko 00260: Glycine, serine and threonine metabolism;
358248990	ko04141: Protein processing in endoplasmic reticulum;	359806310	Ko 05134: Legionellosis
359806527		359807483	Ko 00630: Glyoxylate and dicarboxylate metabolism;
363806826		363806830	Ko 03010: Ribosome
363807896		363808058	
363814326			
363814461		363814581	Ko 04141: Protein processing in endoplasmic reticulum
44887779	ko00944: Flavone and flavonol biosynthesis	451172647	Ko 04110: Cell cycle;
451172655	ko00480: Glutathione metabolism	451806397	Ko 00630: Glyoxylate and dicarboxylate metabolism;
511093970	ko03013: RNA transport;ko05134: Legionellosis	525507124	Ko 00030: Pentose phosphate pathway;
543866	ko00190: Oxidative phosphorylation;	55976189	Ko 03010: Ribosome
571572553	ko03010: Ribosome	574139401	
576017879	ko04810: Regulation of actin cytoskeleton;	587835077	
587838456	ko03008: Ribosome biogenesis in eukaryotes	587840343	Ko 00020: Citrate cycle (TCA cycle);
587842956	ko00500: Starch and sucrose metabolism	587846204	
587846343	ko00010: Glycolysis / Gluconeogenesis;	587846811	Ko 03410: Base excision repair
587847073		587847103	Ko 00071: Fatty acid metabolism;
587847762		587850165	Ko 00010: Glycolysis / Gluconeogenesis;
587850239	ko04626: Plant-pathogen interaction;ko05145: Toxoplasmosis	587850936	
587851024	ko04111: Cell cycle - yeast	587854388	
587854655	ko00020: Citrate cycle (TCA cycle);	587854846	
587854859	ko00905: Brassinosteroid biosynthesis	587854939	
587855105		587856057	
587859860		587860575	Ko 00010: Glycolysis / Gluconeogenesis
587861395	ko00250: Alanine, aspartate and glutamate metabolism;	587863624	
587863779		587863998	Ko 03010: Ribosome
587863999	ko00230: Purine metabolism;	587864131	Ko 00020: Citrate cycle (TCA cycle);
587864869	ko00030: Pentose phosphate pathway	587865279	Ko 00330: Arginine and proline metabolism
587865775	ko03050: Proteasome	587865857	
587866253		587866403	Ko 03018: RNA degradation;
587866816	ko00051: Fructose and mannose metabolism	587867295	

Continued…

Accession	KEGG_PATHWAY	Accession	KEGG_PATHWAY
587868296		587868793	Ko 03040: Spliceosome;
587873236	ko03040: Spliceosome;	587873520	
587874690		587876215	Ko 00020: Citrate cycle (TCA cycle);
587876490	ko00010: Glycolysis / Gluconeogenesis;	587876509	Ko 00190: Oxidative phosphorylation;
587876969	ko00190: Oxidative phosphorylation	587876971	Ko 04141: Protein processing in endoplasmic reticulum;
587876975	ko04141: Protein processing in endoplasmic reticulum;	587877177	Ko 00010: Glycolysis / Gluconeogenesis
587877278		587877585	
587883567	ko00010: Glycolysis / Gluconeogenesis;	587884390	Ko 00270: Cysteine and methionine metabolism;
587884824	ko03018: RNA degradation;	587885625	Ko 04612: Antigen processing and presentation
587886263		587886901	Ko 00010: Glycolysis / Gluconeogenesis;
587887488	ko00030: Pentose phosphate pathway;	587887494	Ko 00330: Arginine and proline metabolism;
587888973		587889431	Ko 00290: Valine, leucine and isoleucine biosynthesis;
587891461		587892622	Ko 00970: Aminoacyl-tRNA biosynthesis
587892913		587892931	
587894021		587894729	Ko 00230: Purine metabolism;
587894874	ko00020: Citrate cycle (TCA cycle);	587898318	Ko 00040: Pentose and glucuronate interconversions;
587898342	ko00020: Citrate cycle (TCA cycle);	587898666	Ko 00600: Sphingolipid metabolism
587899465	ko00010: Glycolysis / Gluconeogenesis;	587899946	
587899953	ko00061: Fatty acid biosynthesis	587906494	Ko 00040: Pentose and glucuronate interconversions;
587906777	ko00620: Pyruvate metabolism;	587906930	
587906940		587907928	Ko 00250: Alanine, aspartate and glutamate metabolism;
587908676	ko00480: Glutathione metabolism	587909106	Ko 03010: Ribosome
587909253		587909774	Ko 05168: Herpes simplex infection;
587910121		587910696	
587913327	ko04145: Phagosome;	587913704	Ko 00600: Sphingolipid metabolism
587913878		587914006	Ko 00020: Citrate cycle (TCA cycle);
587914169	ko00906: Carotenoid biosynthesis	587915213	Ko 00030: Pentose phosphate pathway;
587916056		587918941	
587923270	ko03040: Spliceosome;	587924597	Ko 00051: Fructose and mannose metabolism
587925734		587927232	Ko 03060: Protein export;
587927413		587929519	ko00010: Glycolysis / Gluconeogenesis;
587929625	ko00053: Ascorbate and aldarate metabolism;	587930186	ko03010: Ribosome
587933178		587936284	ko00630: Glyoxylate and dicarboxylate metabolism
587938007		587938205	ko00020: Citrate cycle (TCA cycle);
587938261		587938464	ko00250: Alanine, aspartate and glutamate metabolism;
587939750	ko04626: Plant-pathogen interaction;	587943412	
587943652		587945742	
587945934	ko03050: Proteasome	587948640	
587948991	ko00010: Glycolysis / Gluconeogenesis;	587949150	
587949519	ko00230: Purine metabolism;	587984442	ko00280: Valine, leucine and isoleucine degradation
590000459	ko00190: Oxidative phosphorylation	591403338	ko00360: Phenylalanine metabolism;
593137736		593194039	ko04141: Protein processing in endoplasmic reticulum;
593261812		593262020	ko00020: Citrate cycle (TCA cycle);
593262820		593264720	ko00020: Citrate cycle (TCA cycle);
593266372	ko00250: Alanine, aspartate and glutamate metabolism;	593267439	
593268189	ko04110: Cell cycle;	593268257	
593268449	ko00620: Pyruvate metabolism	593268457	
593280979	ko03008: Ribosome biogenesis in eukaryotes;	593304774	ko03050: Proteasome
593328014	ko04141: Protein processing in endoplasmic reticulum	593328016	ko04141: Protein processing in endoplasmic reticulum
593328228		593328380	ko04146: Peroxisome;
593328570	ko00071: Fatty acid metabolism;	593329699	ko00330: Arginine and proline metabolism;
593330249		593330279	
593330813		593330981	

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Accession	KEGG_PATHWAY	Accession	KEGG_PATHWAY
593331111	ko00010: Glycolysis / Gluconeogenesis;	593331416	
593331596	ko03040: Spliceosome;	593331646	
593331740	ko03013: RNA transport;ko05162: Measles	593332309	
593488621		593489739	ko00860: Porphyrin and chlorophyll metabolism;
593490055	ko00051: Fructose and mannose metabolism;	593490213	
593495777	ko00040: Pentose and glucuronate interconversions;	593555864	ko04141: Protein processing in endoplasmic reticulum;
593584096	ko03050: Proteasome	593662583	
593685749	ko03010: Ribosome	593685795	ko03010: Ribosome
593686200	ko03010: Ribosome	593686524	
593686696		593686914	
593687567	ko03050: Proteasome;	593687701	ko00980: Metabolism of xenobiotics by cytochrome P450;
593689072		593689084	ko03050: Proteasome
593689546		593689589	ko00071: Fatty acid metabolism;
593689643	ko03018: RNA degradation;	593690264	
593692228		593692574	ko00061: Fatty acid biosynthesis
593692678		593693290	ko00053: Ascorbate and aldarate metabolism;
593694040	ko04130: SNARE interactions in vesicular transport	593694544	ko04626: Plant-pathogen interaction
593694805	ko00010: Glycolysis / Gluconeogenesis;	593695565	
593695847	ko00061: Fatty acid biosynthesis;	593696111	
593697406	ko00270: Cysteine and methionine metabolism;	593697474	ko00010: Glycolysis / Gluconeogenesis;
593697638		593698557	ko00290: Valine, leucine and isoleucine biosynthesis;
593700455		593700467	
593700615	ko00564: Glycerophospholipid metabolism;	593700677	
593700899	ko00230: Purine metabolism;	593701185	ko00020: Citrate cycle (TCA cycle);
593701497	ko00010: Glycolysis / Gluconeogenesis;	593702173	ko00270: Cysteine and methionine metabolism;
593702313	ko00010: Glycolysis / Gluconeogenesis;	593702733	ko03050: Proteasome
593702772	ko03013: RNA transport;	593702904	ko04145: Phagosome;ko04540: Gap junction;
593731242		593703537	ko04141: Protein processing in endoplasmic reticulum;
593781167	ko00270: Cysteine and methionine metabolism	593782269	ko00071: Fatty acid metabolism;
593783183	ko03013: RNA transport	593783253	
593783685	ko00480: Glutathione metabolism	593783803	
593784739	ko00040: Pentose and glucuronate interconversions;	593784835	
593784967	ko00620: Pyruvate metabolism	593785081	
593785181		593785995	ko00020: Citrate cycle (TCA cycle);
593786271	ko03050: Proteasome	593787061	ko00061: Fatty acid biosynthesis
593787162		593787628	
593789646	ko00230: Purine metabolism;;	593789662	ko00020: Citrate cycle (TCA cycle);
593792996		593793204	
593794127	ko03010: Ribosome	593794539	ko00620: Pyruvate metabolism;
593794709		593794935	ko05162: Measles
593795400	ko03050: Proteasome	593795810	
593796202	ko00010: Glycolysis / Gluconeogenesis	593796684	ko00010: Glycolysis / Gluconeogenesis;
593796722		593797052	
593797098		593797226	ko04141: Protein processing in endoplasmic reticulum
593797396		593798668	
593798726		593799236	ko05034: Alcoholism;
593799602	ko00010: Glycolysis / Gluconeogenesis;	593799835	
593800193		593800345	ko05110: Vibrio cholerae infection;
593801376	ko00010: Glycolysis / Gluconeogenesis;	593801554	ko00260: Glycine, serine and threonine metabolism;
6014890		62286560	ko04020: Calcium signaling pathway;
62286642	ko05034: Alcoholism;	6831665	ko03010: Ribosome
75303166	ko00941: Flavonoid biosynthesis;		

RESULTS

Proteins were digested and annotated by shotgun method, a total of 288 non-redundant proteins of hazelnut were identified by searching the databases of *Corylus heterophylla Corylus mandshurica, Juglans mandshurica, Pinus koraiensis* in NCBI (table 1).

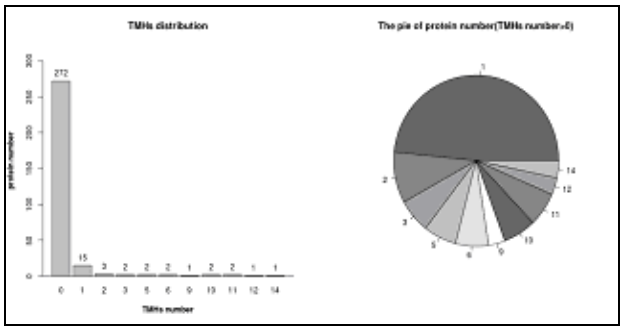

Fig. 2: Transmembrane regions

The theoretical physico-chemical property of hazelnuts by Shotgun

The protein properties, including isoelectric point (pI), molecular weight (MW) and number of residues were calculated by PEPSTATS in EMBOSS according to protein amino acid sequence (Ash burner *et al*, 2000). The grand average hydrophobicity (GRAVY) values were calculated as the arithmetic mean of the sum of the hydropathic indics of each amino acid (Roger *et al*, 2000). Protein transmembrane (TM) regions were predicted with TMHMM (version 2.0). The total number of trans membrane helices (TMHs) of predicted per sequence was reported.

The protein pI was between 4 and 11, with 85 proteins (28.1%) distributed within the range of pI 5-6 (fig. 1). Furthermore, approximately 92 proteins (30.3%) with lowwer pI (less than 6), 237 proteins (78.2%) were mainly distributing in a range of 10-70 kDa (fig. 1). 98 proteins (32.3%) with the number of residues between 200-400 and 88 proteins (29.0%) with the number of residues between 400-600, fig. 2 shows that 31 proteins (10.2%) have trans membrane regions.

Functional categories of hazelnut proteins by gene ontology

To further understand the functions of the proteins identified in this study, we queried all protein sequences against the Uni Prot (SWISS-Prot and TREMBL) (Release 2014_02) by blast (version 2.2.23+) to find proteins which similarity over 30%, used Hit tool to infer protein function. As a result, 288 proteins were annotated and corresponded to GO number, and then classified into biological process, cellular component and molecular function in level 2 according to GO hierarchy and selected categories in generic goslim. Recursive statistical was based on GO hierarchy. in statistical analysis.

Proteins corresponding to GO number are as follows:

Table 2: Unique GO Number of proteins

Protein Number	Unique GO number
243	214
248	226
167	66

Biological process ontology of hazelnut protein

For the biological process ontology, 243 proteins of hazelnut were involved in cellular process (GO: 0009987), metabolic process (GO: 0008152), single-organism process (GO: 0044699). Less commonly, the proteins were related with biological regulation (GO: 0065007), localization (GO: 0051179), developmental process (GO: 00332502), multicellular organismal process (GO: 0032501), cellular component organization or biogenesis (GO: 0071840), multi-organism process (GO: 0051704), reproduction (GO: 0000003), and so on. Most of the cellular and metabolic processes were about synthesis and degradation of small molecules, especially catabolic process, cellular nitrogen compound metabolic.

Cellular components ontology of hazelnut proteins

Gene ontology provides a structured and controlled vocabulary to describe where a gene product is active in the cell (Dyrlov *et al*, 2004). Hazelnut proteins were annotated as being related to cellular components. Of these proteins, 157 occur in the cell (GO:0005623) and 113 in the organelle (GO: 0043226), with the remainder in the membrane (GO: 0016020), macromolecular complex (GO: 0032991), membrane-enclosed lumen (GO: 0031974) and extra cellular region (GO: 0005576).

Molecular function ontology of hazelnut protein

In the molecular function ontology, 12 subcategories were assigned, of which the activities of catalytic (GO: 0003824, 183 proteins, 73.8% of annotated peptides) and binding activity (GO: 0005488,170 proteins, 68.5%) were the major categories. Most of the mapped catalytic activity were oxidoreductase activity, kinase activity, peptidase activity and, to a lesser extent, isomerase activity. The proteins in the binding activity group also can be classified into subgroups based on the specific functions. Most of proteins are related to ion binding and RNA binding. The groups with much fewer terms included DNA binding and unfolded protein binding. Besides that 12 subcategories also includes structural molecule activity, antioxidant activity, transporter activity, electron carrier activity, enzyme regulator activity, nucleic acid binding transcription factor activity, receptor activity and nutrient reservoir activity.

CONCLUSION

A total of 303 proteins of Changbai Mountain hazelnut were identified and annotated by shotgun method. Gene ontology annotation showed that hazelnut proteins had

catalytic activity, binding activity, transshipment, antioxidant activity etc. Besides that, hazelnut proteins were involved in cellular process, metabolic process, single-organism process, biological regulation, localization, developmental process. Based on shotgun proteomics strategies, functional verification could be conducted and it will provide important references for development of edible and medical value of hazelnut protein and offer theoretical basis for utilization of natural resources of Changbai Mountain in Jilin province.

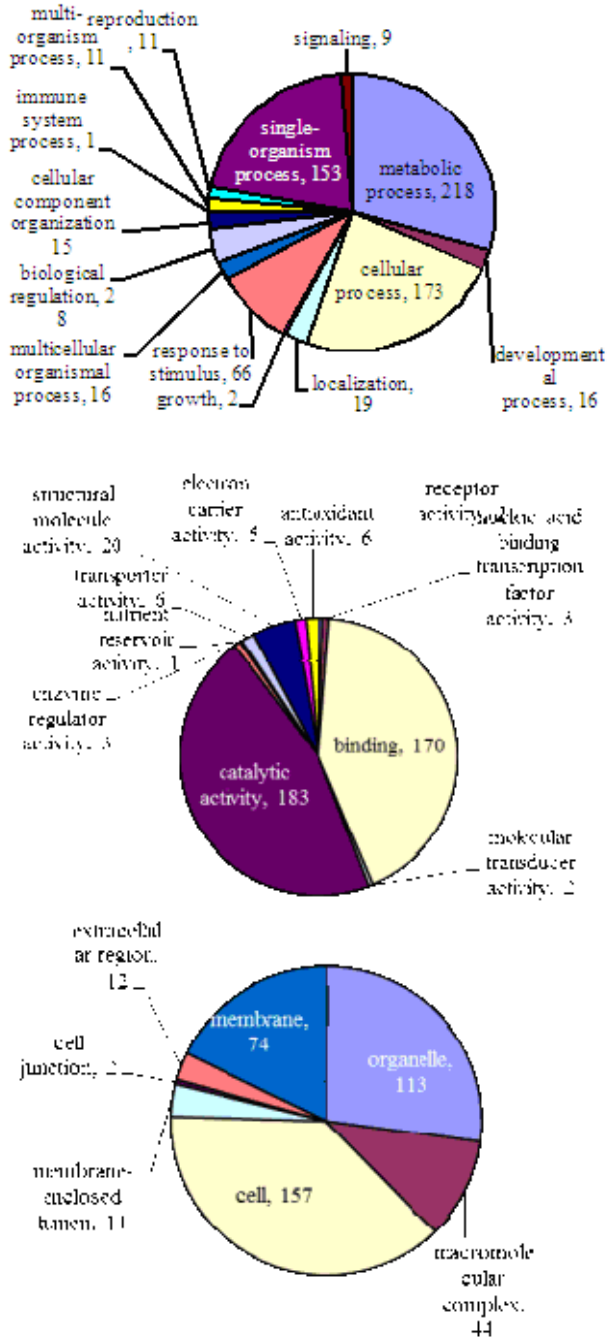

Fig. 3: Classification of GO Annotation

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