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Research Article

Differences in proteomic and peptide profiles of whey protein by acid curd and enzyme curd process from bovine milk

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Abstract: Whey protein, an essential component of milk, possesses a rich repertoire of bioactive proteins with unique nutritional and functional properties. Employing liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), we conducted a comparative analysis of whey proteins derived from enzyme curd Esprion300HS (E300HS) and acid curd Textrion PROGEL 800 (TP800) methods. Our investigation identified 216 proteins, of which 32 exhibited differential expression between E300HS and TP800. Notably, E300HS displayed significantly higher levels of calcium-binding protein, lactoperoxidase, and lactadherin compared to TP800. Peptide profiling revealed 162 peptides, with 12 upregulated and 14 downregulated in E300HS relative to TP800. Furthermore, E300HS exhibited abundant lactoferrin-derived peptides, while TP800 displayed a higher prevalence of casein-like peptides. These proteins and peptides encompass a broad range of biological functions, including anticancer, antimicrobial, and bone remodeling activities. Our findings underscore the vast potential of whey protein in food industry applications and its significance in cancer research, bone development, and age-related degenerative diseases.

Keywords: proteomic; whey protein; peptide; active ingredient

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1 Introduction

Whey protein, derived from the copious liquid fraction of whey produced during cheese production by the enzyme curd and acid curd method, undergoes meticulous refinement processes involving separation, concentration, and drying[1]. Acid curd whey protein concentrate (WPC) is obtained by precipitating casein at pH 4.6 using starter cultures or acids, without the involvement of rennet[2]. It is characterized by a high protein content and relatively low mineral content[1–2]. In contrast, enzyme curd WPC is produced by enzymatically hydrolyzing casein, resulting in protein fragments with smaller molecular weights[3]. These protein concentrates offer unique nutritional profiles and may have applications in various industries, including sports nutrition, dietary supplements, and functional foods. Understanding their manufacturing processes and functional properties is essential for optimizing their utilization and developing innovative protein-based products[4–5]. The bioactive constituents of whey protein, including β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulin, lactoferrin (LF), lactoperoxidase (LP), and minor components, contribute to its health benefits and make it a vital source of high-quality protein for optimal bodily function and well-being[6–7]. Whey protein is particularly rich in essential amino acids, such as leucine, valine, isoleucine, and cysteine, which plays critical roles in metabolism, neurological function, and homeostasis[5]. Leucine, in particular, serves as a crucial signaling molecule that regulates skeletal muscle protein anabolism[8]. Additionally, the growth factors and cytokines present in whey protein have been shown to stimulate cell proliferation and growth, highlighting its significance in various biological processes[5].

While the nutritional and biological properties of whey protein have been extensively reported, its activities are primarily attributed to bioactive peptides encoded within the protein sequence[9–10]. These peptides can be released through enzymatic hydrolysis during gastrointestinal digestion or food processing[11]. Depending on their amino acid sequences, these peptides exhibit various functional properties, including mineral binding, immunomodulatory, antimicrobial, antioxidant, antithrombotic, and antihypertensive effects[12]. Zhong et al.[7] and Gilmartin et al.[8] conducted a study on the biological activity of hydrolysates from various sources of whey protein using an *in vitro* simulated human digestion model. They discovered that the hydrolysates exhibited significantly higher levels of biological functions, such as antioxidant, antibacterial (*Listeria monocytogenes* and *E. coli*), anti-inflammatory, and immune regulation, compared to the original protein[8,13]. In recent years, Qi et al.[10] have employed liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), in combination with molecular docking technology, to elucidate small molecule peptides within whey protein isolates that undergo degradation by pepsin-pancreatin. Notably, these peptides, including PEW, LLW, GINY, and DQW, exhibit the remarkable capability to transverse the intestinal barrier and actively participate in the modulation of metabolic pathways governing fatty acids and uric acid. Consequently, they hold great promise in alleviating the incidence of metabolic disorders, such as diabetes, obesity, hyperuricemia, and hypertension, by virtue of their multifaceted regulatory

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effects[9–10]. Therefore, food production process could affect the ultimate bioactive characteristics of the peptides in the food system. However, there are little research about the effects of processing technology on bioactive peptides in foods.

Extensive research has focused on the proteomic and peptide profiles of bovine whey protein, there is a paucity of studies investigating the differences in composition and biological activities between whey protein derived from acid curd and enzyme curd methods[1,14]. This study aims to address this research gap by employing label-free methods to perform a comparative analysis of the proteomic and peptide profiles of whey protein obtained through acid curd Textrion PROGE 800 (TP800) and enzyme curd Esprion300HS (E300HS) processes. Through LC-ESI-MS/MS analysis, digested proteins and peptides were identified, and their relative abundances were carefully examined. Additionally, bioinformatics analysis was employed to gain insights into the potential biological functions of whey protein and discern the specific variances between acid curd and enzyme curd products sourced from bovine. This study expands our understanding of the whey proteome while shedding light on the potential biological activities associated with whey protein.

2 Materials and methods

2.1 Materials and chemicals

The E300HS (32% protein, 8% ash, 48% lactose, 2% fat) and TP800 (80% protein, 5% ash, 7% lactose, 5% fat) were obtained from DMW International B.V., Netherlands. E300HS was produced using the enzyme curd method: fresh milk heated to 45–50 °C, mixed with a coagulating enzyme, incubated, and the resulting curds were separated from the whey and refrigerated. TP800 was obtained through the acid curd method: fresh milk heated to 85–90 °C, lactic acid added, incubated, and the curds and whey were separated through filtration, with the liquid whey being refrigerated. Chemicals and reagents used were purchased from Sigma (Saint-Louis, MO, USA).

2.2 Whey protein compositional analysis

Whey protein composition was analyzed using the Kjeldahl method. The whey protein sample was digested in concentrated sulfuric acid (10 mL/g sample) at 420 °C for 2 h. The released nitrogenous compounds were converted into ammonium sulfate using sodium hydroxide and a catalyst mixture. Steam distillation for 30 min separated the ammonium sulfate, collected in a flask with 25 mL standard acid solution. Excess acid was titrated with 0.1 mol/L sodium hydroxide. Non-casein nitrogen (NCN), casein nitrogen (CN), and non-protein nitrogen (NPN) proportions were calculated. The Kjeldahl method was performed in triplicate, and statistical analysis assessed significant differences between samples.

2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Whey protein samples (E300HS and TP800) were subjected to SDS-PAGE using a Bio-Rad electrophoresis system. Samples (1 mg) were suspended in reducing buffer, denatured, and loaded onto a 12% polyacrylamide gel. The gel was stained with Coomassie brilliant blue R-250 and destained with a methanol and glacial acetic acid solution (9:2, *V*/*V*). Gel images were captured using an LKB 2400 GelSCan XL gel imaging system (Bio-Rad, USA).

2.4 Identification of protein and pepide profiles

2.4.1 LC-ESI-MS/MS analysis by Q exactive

The whey protein components were further analysed with capillary LC-ESI-MS/MS based on the method of Li et al.[12]. The peptides were separated on Agilent 1200 chromatographic system (Agilent Technologies, Wilmington, DE) after concentrating and desalting the products, which conducted by Thermo Scientific EASY C_{18} column (2 cm × 100 μm, 5 μm) and (75 μm × 100 mm, 3 μm), at a flow rate of 300 nL/min. Solvents A and B for chromatography were 0.1% (*V*/*V*) formic acid-water and 0.1% (*V*/*V*) formic acid-acetonitrile water (84% of acetonitrile), respectively. The gradient elution was: 0 min, 0% B; 50 min, 35% B; 55 min, 100% B; 60 min, 100% B.

The separated peptide samples were added on the mass spectrometer with positive ion mode for 1 h and MS range of *m*/*z* 300–1 800. The 20 most intense ions in each MS survey scan were subjected to MS-MS by higher-energy collisional dissociation (HCD). Dynamic exclusion duration was 60 s and the automatic obtaining control target value was 3E6. 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. Normalized collision energy was 30 eV and the underfill ratio was defined as 0.1%. The instrument was run with peptide recognition mode enabled.

2.4.2 Sequence database searching and data analysis

MASCOT engine and Proteome Discoverer 1.3 were applied for search and analysis of MS-MS spectra based on the uniprot_bovine.fasta database (downloaded on 2017-07-25, http://www.uniprot.org). The search parameters of Mascot and the analysis parameters of protein identification were carried out according to the method of Guan et al.[15] and Li et al.[16] with slight modification.

2.5 Bioinformatics analysis

2.5.1 Characterization of protein and peptide properties

The Protparam tool (https://web.expasy.org/protparam) was employed for online analysis of protein and peptide attributes, including molecular weight, theoretical isoelectric point (pI), amino acid composition, and stability.

2.5.2 Gene ontology (GO) analysis

GO analysis was conducted to explore the biological characteristics of differentially expressed proteins. GO analysis was performed using the UniProt (https://www.uniprot.org/) and DAVID database (https://david.ncifcrf.gov/) with default settings and significance thresholds. Enriched biological processes (BP), molecular functions (MF), and cellular components (CC) associated with the differentially expressed proteins were annotated and visualized.

2.6 Statistical analysis

Results were analyzed statistically using the SPSS 19.0 for independent samples *t*-test. For all statistical analyses, a value of $P < 0.05$ for the comparisons was considered to represent a significant difference between groups.

3 Results and discussion

3.1 The composition of E300HS and TP800

Protein content is a crucial determinant of the functional properties and nutritional value of whey[1]. In this study, we examined the protein composition of whey obtained through acid and enzyme

coagulation processes. E300HS and TP800 exhibited distinct total protein contents of 29.37% and 76.66%, respectively (Table 1). Surprisingly, both samples showed similar whey protein compositions, representing the majority (> 80%) of the total protein. However, notable differences in NPN content were observed, with E300HS containing 0.37% and TP800 containing only 0.03% of the total protein. SDS-PAGE analysis unveiled four prominent protein bands in the 25 to 116 kDa range (Figure 1), corresponding to major whey proteins, namely β-lactoglobulin (β-Lg, 18 kDa), α-lactalbumin (α-La, 14.1 kDa), bovine serum albumin (BSA, 67 kDa), and LF (80 kDa)[6,17].

Table 1 Protein composition in two whey protein samples.

Item	E300HS	TP800
Total protein (%)	29.37	76.66
NPN/total protein (%)	0.37	0.03
Whey protein/total protein (%)	81.78	85.57
Casein/total protein (%)	14.20	6.50

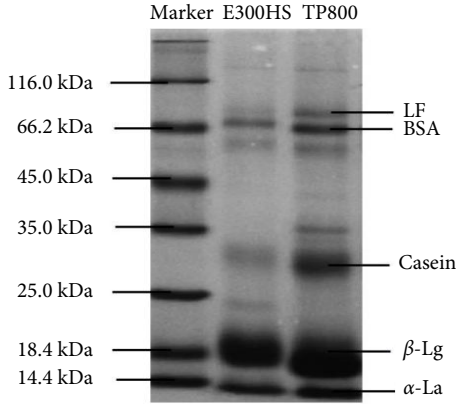


Figure 1 SDS-PAGE analysis of E300HS and TP800 whey protein composition.

Specifically, our focus was on comparing the relative abundance of casein, BSA, and LF within the 20 to 65 kDa range in whey protein derived from acid curd and enzyme curd processes. The results demonstrated significant variations in protein composition between the two variants. Enzyme curd exhibited lower levels of casein and LF in the whey protein compared to acid curd. This difference can be attributed to the specific enzymatic actions involved in the enzyme curd process[1]. The enzymes used in enzyme curd selectively target and degrade casein and LF, facilitating their breakdown and subsequent release into the whey[2]. In contrast, the acid environment in acid curd hinders the degradation and release of these proteins, potentially leading to their aggregation and reduced solubility in the whey[3]. Furthermore, enzyme curd led to reduced levels of BSA in the whey protein compared to acid curd. The enzymes used in enzyme curd selectively degrade BSA, resulting in a decreased release of BSA into the whey[1,3]. Additionally, the whey fraction contained several unidentified proteins within the 20 to 65 kDa range, indicating the presence of other components contributing to the overall protein composition. Further investigations are necessary to identify and characterize these unknown proteins. These findings emphasized the influence of coagulation mechanisms and environmental conditions on the composition of whey protein. A comprehensive understanding of the specific enzymatic actions, the effects of acidic conditions, and the nature of unidentified proteins within whey protein can inform process optimization and enhance the quality and functionality of whey protein in the dairy industry.

3.2 Identification of whey protein and its peptides composition

The whey protein samples were further analyzed using label-free and tryptic peptide sequence matching with MASCOT and UniPort[16]. Two hundred and sixteen proteins and 162 peptides were identified and quantified through LC-ESI-MS/MS. Among these, 202 and 192 proteins, 154 and 129 peptides were involved in E300HS and TP800, respectively. The higher amount of proteins and peptides in E300HS is due to the enzymatic hydrolysis involved in its manufacturing process. The E300HS is crafted using enzyme-based curd cheese whey, which is capable of hydrolyzing proteins and releasing peptides, which can be achieved through the use of chymosin as well as starter or microbial enzymes[18]. Meanwhile, the TP800 is created using acid curd technology, whereby the primary source of peptides in this particular sample derives from the acid hydrolysis of proteins.

3.3 GO analysis

GO analysis was performed to obtain the biological processes and molecular functions of differentially expressed proteins between E300HS and TP800. The percentages of MF, CC, and BP terms involved in differentially expressed proteins were presented in Figure 2.

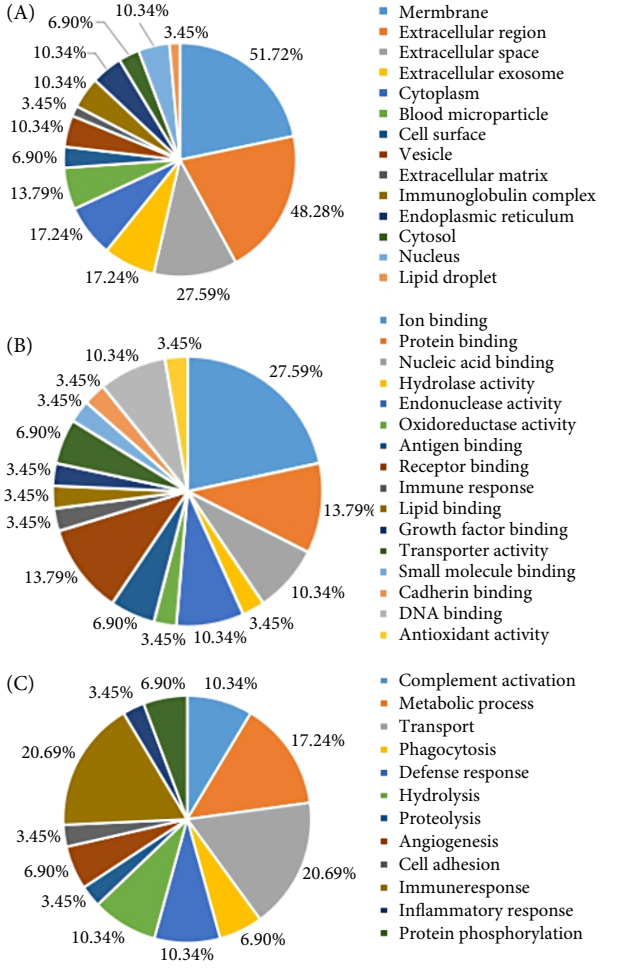


Figure 2 GO classification results of differential expressed proteins between E300HS and TP800. (A) Annotation of CC; (B) Annotation of MF; (C) Annotation of BP.

The analysis of CC revealed that whey proteins predominantly localized to the membrane (51.72%) and extracellular region (48.28%). This distribution implies their pivotal roles in cellular interactions and responses to the external environment. Furthermore, these proteins were also found in the extracellular space (27.59%), cytoplasm (17.24%), blood microparticles (13.79%), vesicles (10.34%), and endoplasmic reticulum (10.34%), indicating their involvement in diverse cellular compartments.

Regarding MF, ion binding emerged as the primary function (27.59%), suggesting the participation of these proteins in ion transport and intracellular ion homeostasis. Notable functions included protein binding (13.79%), receptor binding (13.79%), nucleic acid binding (10.34%), DNA binding (10.34%), transporter activity (6.90%), antigen binding (6.90%), oxidoreductase activity (3.45%), and hydrolase activity (3.45%). These varied functions highlight the versatility and functional diversity of the differentially expressed proteins.

In terms of BP, transport (20.69%) and immune response (20.69%) were identified as the most prevalent processes. These findings indicated the crucial roles of these proteins in cellular transport processes and immune system regulation. Additionally, differentially expressed whey proteins were involved in metabolic processes (17.24%), complement activation (10.34%), defense response (10.34%), hydrolysis (10.34%), and angiogenesis (6.90%), underscoring their participation in vital biological activities.

These results provide valuable insights into the functional characteristics of differentially expressed proteins (E300HS vs. TP800), highlighting their significance in cellular processes and potential implications in the field of food science.

3.4 Differences in proteomic profiles of E300HS and TP800

The analysis of differential expressed proteins and peptides by the methods of LC-ESI-MS/MS was conducted. Results indicated that 32 proteins and 33 peptides exhibited a significant difference in normalized volume between E300HS and TP800 ($P < 0.05$). Out of these, 18 proteins and 12 peptides were up-regulated, while 14 proteins and 21 peptides were down-regulated, respectively.

3.4.1 *Analysis of significant up-regulated proteins between E300HS and TP800*

To compare the composition and functional properties of whey protein derived from different processing sources and determine the effects of processing on whey protein, we conducted a study comparing the relative abundance of E300HS and TP800. Table 2 presented the 18 proteins that were significantly up-regulated in E300HS compared with TP800. Additionally, we found that high abundance proteins including peroxidase (0.797%), β-2-microglobulin (0.743%), LF (0.740%), perilipin-2 (0.111%), and immunoglobulin (0.184%), were significantly up-regulated in E300HS compared with TP800.

LP is a high-value minor whey protein that has recently received extensive attention from research scientists and industry due to its multiple functions, including antibacterial, antiviral, anticancer, and antioxidation. LP also has potential therapeutic applications[19]. It can catalyze the oxidation of thiocyanate ion (SCN^-) by hydrogen peroxide, hypophosphite ($OSCN^-$), and hypothiocyanate (OSCNH) with broad antimicrobial activity[20].

Immunoglobulin is one of the most important biologically active proteins in milk which plays an important role in enhancing the body's immunity, preventing the invasion of bacteria and viruses, activating complement, neutralizing toxins, and killing tumor cells[21]. Zhong et al.[7] and Ohnuki et al.[22] found that diets supplemented with bovine immunoglobulin G (IgG) and ovalbumin (OVA) alone can induce superoxide content of interleukin-12 cluster of differentiation 1 lymphoid cell subset (IL-12 CD1lb) cells and macrophages increased in the spleen, the resultsindicated that IgG can stimulate innate cellular immune system to improve digestion and clearance of pathogens.

β-2-Microglobulin is a non-covalently associated light chain of the major histocompatibility complex class I (MHCI) glycoprotein. It is present on the surface of all nucleated cells and in various physiological fluids. It shares homology with the constant regions of

Table 2 Significant up-regulated proteins in E300HS compared with TP800.

No.	Accession	Protein name	E300HS/TP800	Molecular weight (kDa)	pI
1	Q3ZBZ1	Calcium-binding protein	10.1	45.0	4.79
2	F1MWK1	Transient receptor potential cation channel subfamily M member 6	6.5	99.3	7.84
3	Q3MHX6	Protein OS-9	5.8	75.8	4.84
4	P80025	LP	5.7	80.6	8.83
5	Q3SYR8	Immunoglobulin J chain	5.7	17.9	5.07
6	Q2UVX4	Complement C3	4.7	187.3	6.41
7	Q95114	Lactadherin	4.0	47.4	6.81
8	Q9TUM6	Perilipin-2	3.9	49.4	8.72
9	Q0P569	Nucleobindin-1	3.4	55.0	5.11
10	Q862Q3	β-2-Microglobulin	3.0	11.0	7.08
11	G3N342	Immunoglobulin heavy constant epsilon	2.7	54.9	6.55
12	F1MGR1	Sodium/nucleoside cotransporter	2.6	77.3	7.20
13	Q148D9	Cellular repressor of E1A-stimulated genes 1	2.4	24.0	6.89
14	A6QNW7	CD5L protein	2.2	50.2	5.24
15	Q2KIT0	Protein HP-20 homolog	2.2	20.7	8.85

the IgG light and heavy chains. By binding with peptides, it regulates the expression of MHC class I proteins[23].

Above all, the proteins that were up-regulated in the E300HS and TP800 samples were mainly involved in biological processes, such as antibacterial reactions, immune reactions, and metabolism. Moreover, they can also aid in the growth of the body's immune system and prevent the invasion of bacteria and viruses. As a result, due to its biological functionality, the E300HS is more appropriate for newborns and teenagers than TP800.

3.4.2 Analysis of significant down-regulated proteins between E300HS and TP800

Four high-abundance proteins were identified in whey, including osteopontin (2.67%), angiogenin-2 (0.09%), angiogenin-1 (1.31%), and ribonuclease 4 (0.61%), all of which were among the 14 down-regulated proteins identified between E300HS and TP800 (Table 3).

Osteopontin (OPN) is a highly phosphorylated sialoprotein that plays a crucial role in bone remodeling, immune response, and inflammatory response[24]. It has been found that OPN promotes the migration of bone marrow mesenchymal stem cells through the focal adhesion kinase (FAK) /extracellular signal-regulated kinase 1/2 (ERK1/2) signal transduction pathway, which particicpates in wound healing[25]. Moreover, OPN also facilitates vascular smooth muscle cell proliferation via 4-hydroxynonenal (HNE)-mediated activation of activator protein 1 (AP-1) and CCAAT/enhancer-binding protein beta (C/EBPβ) pathways[26]. In lung cancer cells, OPN binds αvβ3 integrin, which in turn stimulates the phosphorylation of FAK and nuclear factor-κB (NF-κB) through phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) or ERK signaling pathway, leading to altered cell migration[27].

Ribonuclease-4 and angiogenin belong to the superfamily of hydrolyzable endonuclease[28], which has been found to interact efficiently with both endothelial and smooth muscle cells, triggering some cellular responses such as cell migration, invasion, proliferation, and tubular formation. This characteristic is related to its strong pro-angiogenic nature[29]. Conversely, angiopoietin-1 can activate the intracellular PI3K pathway, enhancing the anti-apoptotic factor Bcl-2 expression, and successfully thwarting H_2O_2-induced myoblast apoptosis[30–31].

Taken together, these results demonstrated that the biological processes involving down-regulated proteins in E300HS and TP800 are associated with bone remodeling, cell proliferation, cell migration, inflammatory responses. Additionally, they may exert a significant role in preventing aging-related diseases, such as osteoporosis, amyotrophic lateral sclerosis, and cancer[27–28]. Therefore, compared with E300HS, the biological function of TP800 appears to be more suitable for the elderly population.

3.5 Differences in peptidomics profiles of E300HS and TP800

3.5.1 Analysis of significant up-regulated peptides between E300HS and TP800

Bioactive peptides derived from whey proteins are involved in a wide range of physiological activities, including antioxidant activity, immuno-stimulating functions, and anti-inflammatory effects[5,32]. In order to investigate the effect of production technology on the components and functions of whey protein peptides, we analyzed the differences in peptides obtained by acid curd and enzyme curd production processes using LC-ESI-MS/MS. The up-regulated peptides in E300HS compared with TP800 were shown in Table 4. Twelve significant up-regulated peptides were identified, and it is noteworthy that the peptides derived from LF (No. 1) and lactadherin (No. 4–6) were highly abundant peptides in E300HS.

Although no reports have been made regarding these unfamiliar peptides, several studies have investigated the ability of LF peptides to inhibit bacterial growth through the addition of these hydrolysates to bacterial cultures following hydrolysis by gastric, rennet, or microbial coagulation enzymes. These studies have demonstrated that LF peptides possess significant antibacterial activity[33]. Moreover, potential biological functions of LF hydrolysates were identified in the regulation of human bone health[34].

Lactadherin is a glycoprotein that exhibits hydrophilic properties and commonly found in milk fat globules. It can be hydrolyzed by gastric acid or pancreatic enzymes. *In vitro* studies suggested that preheating and demetallization contribute to lactadherin protein

Table 3 Significant down-regulated proteins in E300HS compared with TP800.

No.	Accession	Protein name	E300HS/TP800	Molecular weight (kDa)	pI
1	A1L555	Prosaposin (proactivator polypeptide)	0.498	58.2	5.13
2	Q9MZ06	Fibroblast growth factor-binding protein 1	0.443	26.2	9.40
3	A6QQ20	TGOLN2 protein	0.430	33.4	6.75
4	O02853	Prostaglandin-H2 *D*-isomerase	0.406	21.2	6.43
5	Q56J78	Serum amyloid A protein	0.396	14.7	9.48
6	Q3SX32	Perilipin	0.361	47.6	5.77
7	G3N0V2	Keratin, type II cytoskeletal 1	0.337	63.2	8.59
8	P31096	Osteopontin	0.306	30.9	4.49
9	P80929	Angiogenin-2	0.186	14.5	9.97
10	Q2NKV1	Angiogenin, ribonuclease	0.166	17.0	9.10
11	A7E350	PLG protein	0.155	81.6	8.83
12	Q28078	Epithelial mucin	0.149	20.9	7.06
13	Q2HJI6	Granulin	0.126	63.1	7.81
14	Q58DP6	Ribonuclease 4	0.122	16.9	9.18

Table 4 Significant up-regulated peptides in E300HS compared with TP800.

No.	Sequence	Pepeides source	E300HS/TP800	Molecular weight (Da)	pI	Stability
1	KGSNFQLDQLQGR	LF (f 100–112)	11.42	1 490.6	8.75	Stable
2	VEEAKRKGQETISQLHSAFNLSELARK	Perilipin-2 (f 239–265)	8.42	3 070.5	8.48	Unstable
3	RSIGYDDTDESHCAEHIESRTLAIAR	Perilipin-2 (f 290–315)	8.19	2 946.2	4.92	Unstable
4	KMWVTGVVTQGASR	Lactadherin (f 156–169)	5.08	1 519.8	11.00	Stable
5	VTGIITQGARDFGHIQYVAAYRVAYGDDGVTWTEYKDPGASESK	Lactadherin (f 321–364)	5.06	4 794.2	4.89	Stable
6	WAPELARLHQTGIVNAWTSGNYDK	Lactadherin (f 122–145)	4.38	2 728.0	6.15	Stable
7	DAVTTTVTGAKDSVASTITGVVDR	Perilipin-2 (f 117–140)	3.09	2 364.6	4.43	Stable
8	PIFVPAKARKK	CD5L protein (f 371–381)	2.75	1 254.6	11.27	Unstable
9	SHPSAPPPYEDR	PP1201 protein (f 2–13)	2.20	1 352.4	5.30	Unstable

degradation. Apart from this, lactadherin plays a significant role in numerous physiological and pathological processes, such as phagocytosis, atherosclerosis, angiogenesis, and virus protection[35]. Previous researches indicated that lactadherin activates several signaling pathways, prominently including PI3K/Akt and ERK, to facilitate its effects on cell growth, invasion, metastasis, anti-apoptosis, and angiogenesis[34,36].

The perilipin-2 (No. 2–3) peptide was also present high density in E300HS. Perigrin-2 is a well-known protein found in lipid droplets that covers their surface in all mammalian cells[37]. Perilipin-2 can induce diet-induced obesity, adipose inflammation, and fatty liver disease[37]. Specifically, perigrin-2 prevent the binding of lipase to lipid droplets, thereby slowing down the conversion of triglycerides, which helps regulating lipid storage in cells. The up-regulated peptides are mainly derived from lactotransferrin, lactadherin, and perilipin-2. These peptides are involved in biological processes associated with antibacterial, antiviral protection, and angiogenesis. Furthermore, up-regulated peptides play a crucial role in the development of atherosclerosis, cancer, and obesity-related diseases.

3.5.2 Analysis of significant down-regulated peptides between E300HS and TP800

As shown in Table 5, 21 significant down-regulated peptides were identified between E300HS and TP800. It is noteworthy that TP800 has larger amount of peptide sequences that exhibit significant similarity to casein peptides compared with E300HS.

Table 5 Significant down-regulated peptides in E300HS compared with TP800.

No.	Sequence	Pepeides source	E300HS/P800	Molecular weight (Da)	pI	Stability
1	PVALINNQFLPYPYYAK	κ-Casein (f 47–63)	16.68	2 011.4	8.83	Unstable
2	HAKGRTRTAIRNGQVWEESLKRLRR	LP (f 54–78)	14.63	3 019.5	12.01	Unstable
3	KTKLTEEEKNRLNFLKKISQRYQKFALPQYLKTVYQHQK	α_{s2}-Casein (f 165–203)	11.80	4 869.7	10.08	Stable
4	LHSMKEGIHAQQK	α_{s1}-Casein (f 120–132)	8.03	1 506.7	8.61	Stable
5	YIPIQYVLSR	κ-Casein (f 25–34)	7.98	1 251.5	8.59	Stable
6	YPSYGLNYYQQK	κ-Casein (f 35–46)	6.09	1 523.7	8.38	Unstable
7	NDIKAICEDRNGQPYRGDLRISKSEFQITICK	Angiogenin (f 52–83)	5.45	3 712.2	8.02	Stable
8	TTLSSEAPTTQQLSEYFK	LP (f 36–53)	5.06	2 031.2	4.53	Unstable
9	YIHFLTQHYDAK	Angiogenin (f 7–18)	5.05	1 535.7	6.92	Unstable
10	DKKSCHTAVDR	LF (f 453–463)	4.63	1 259.4	8.21	Stable
11	SCQAQPTTMAR	κ-Casein (f 87–97)	3.75	1 193.4	7.96	Unstable
12	DTNTFIHGNSDDIR	Angiogenin-2 (f 40–53)	3.46	1 604.7	4.41	Stable
13	QLNNKYPDAVATWLKPDPSQK	Osteopontin (f 15–35)	3.33	2 413.7	8.43	Stable
14	RPKHPIKHQGLPQEVLNENLLRF	α_{s1}-Casein (f 1–23)	2.90	2 764.2	9.99	Unstable
15	EKVNELSK	α_{s1}-Casein (f 35–42)	2.73	946.07	6.24	Stable
16	KIEFQSEEQQQTEDELQDK	β-Casein (f 29–48)	2.53	2 352.5	4.00	Unstable
17	GDSVAYGLK	Osteopontin (f 137–145)	2.52	909.0	5.83	Stable
18	HIQKEDVPSER	α_{s1}-Casein (f 95–105)	2.20	1 337.5	5.45	Unstable
19	KNACCSVNTSIEAHK	Folate receptor (f 42–56)	2.18	1 064.8	8.06	Unstable
20	IGACPAAHKPLLGAEK	Prosaposin (f 464–479)	2.10	1 575.9	8.21	Unstable
21	DAIPENLPPLTADFAEDK	Serum albumin (f 301–318)	2.09	1 956.1	3.77	Stable

Casocidin-1 is an antibacterial peptide derived from α_{s2}-casein (f 165–203) and possesses significant antimicrobial activity. Lucia et al.[38] found that both α_{s2}-casein and casocidin-1 can impede the growth of *E. coli* and *Staphylococcus aureus*, thereby inhibiting bacterial growth. Casoxin is a class of opioid-like peptides that originate from bovine κ-casein. Chiba et al.[39] performed *in vivo* antagonism experiments on trypsin-digested products of κ-casein and identified only casoxin-C (YIPIQYVLSR) as exhibiting antagonistic activity. Lahov et al.[40] analyzed the activity of isracidin (RPKHPIKHQGLPQEVLNENLLRF) derived from α_{s1}-casein, and found that isracidin can inhibit the growth of *Staphylococcus aureus* and *Candida albicans*. Donida et al.[41] studied the effect of casein phosphopeptides (CPP) derived from bovine casein and digested by trypsin on human primary osteoblasts *in vitro*, further found that CPP can directly stimulate calcium uptake by cells and regulate cell differentiation by increasing expression and alkaline phosphatase activity.

In summary, the significant down-regulated polypeptides have high similarity to casein peptides, among which a considerable number of peptides exhibited antibacterial and antagonistic effects. Additionally, these polypeptides have been found to be associated with biological processes such as defense response and angiogenesis.

4 Conclusion

In this study, we analyzed whey protein profiles from bovine sources (E300HS and TP800) using LC-ESI-MS/MS. We identified 216 proteins, with 32 showing significant differential expression. E300HS exhibited higher levels of calcium-binding protein, LP, and lactadherin. Peptide profiling revealed 162 peptides, with 12 up-regulated and 14 down-regulated in E300HS. E300HS had abundant LF-derived peptides, while TP800 had more casein-like peptides. These proteins and peptides have diverse biological functions, including anticancer, antimicrobial, and bone remodeling activities. Our findings provide insights into whey proteomics, highlighting the importance of understanding protein compositions for functional utilization. This research expands our knowledge of whey protein and its potential applications in biological processes.

Conflict of interest

The authors declare no conflicts of interest.

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