Chapter

Application of Two-Dimensional Gel Electrophoresis in Combination with Mass Spectrometry in the Study of Hormone Proteoforms

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Abstract

Hormone is a category of important endocrine regulatory proteins in human endocrine systems. Clarification of hormone proteoforms directly leads to understanding of its biological roles. Two-dimensional gel electrophoresis (2DGE) in combination with mass spectrometry (MS) plays important roles in identification of hormone proteoforms such as human growth hormone (hGH) proteoforms and human prolactin (hPRL) proteoforms. This book chapter will review the hormone proteoforms focusing on hGH and hPRL, the methodology of hormone proteoform study, and future perspective of human hormone proteoform study to find biomarkers for in-depth understanding of molecular mechanisms, and individualized and precise diagnosis, therapy, and prognostic assessment of hormone-related diseases.

Keywords: two-dimensional gel electrophoresis, mass spectrometry, tandem mass spectrometry, liquid chromatography, hormone, growth hormone, prolactin, variant, proteoform

1. Introduction

Hormone is a category of important endocrine regulatory proteins in the human endocrine systems. Hormone is a chemical message substance synthesized by highly differentiated endocrine cells and directly secreted into the blood, which has high biological activities and transmits information in the body as a messenger. It is a vital substance in human life and plays important roles in regulating physiological processes such as metabolism, growth, and development.

Notably, human growth hormone (hGH) and human prolactin (hPRL) are two key hormones in human body. The hGH, also known as somatotropin, is produced in the acidophilic somatotroph cells of the anterior pituitary gland and is a 191 amino acid single chain polypeptide, which is released into the blood circulation and takes part in the hypothalamic-anterior pituitary-skeletal muscle axis system to regulate growth and development in human body [1, 2]. The synthesis and release of hGHs are affected by multiple complex feedback mechanisms, and the

major regulators are growth hormone-releasing hormone (GHRH) synthesized in the hypothalamus, somatostatin derived from various tissues of the body, and ghrelin produced in the gastrointestinal tract. The mechanism of the effect of hGH is to directly bind with target organs for response of stimulation or it is indirectly influenced by the role of insulin-like growth factor-1 (IGF-1). The IGF-1 secreted from hepatocytes responds to the binding of hGH to surface receptors. If the Janus activating tyrosine kinases (JAKs) 1 and 2 are activated and bound to the cytoplasmic transcriptions factors STAT1, STAT3, and STAT5, and transported into the nucleus, thus enhanced gene transcription and metabolism would be induced, and the corresponding IGF-1 is generated and released into circulation. IGF-1 affects the growth and metabolism of peripheral tissues, which is able to understand as a combined impact of IGF-1 and hGH [2, 3]. The deficiency of hGH leads to growth deficit in children and the GH deficiency syndrome in adults, conversely, hGH hypersecretion results in gigantism or acromegaly [4–7]. It is obvious that hGH plays an important role in regulation of human life activities. In addition, hPRL should be paid more attention to its relevant functional study. The hPRL, a four long α-helix protein hormone, is mainly secreted by lactotrophs in the anterior pituitary gland except placental PRL [8]. The secretion of hPRL is usually regulated by PRL inhibitors such as dopamine from hypothalamus [9]. The hPRL is a polypeptide hormone with a wide variety of functions such as controls osmotic pressure and vascularization, participates in the immune response, and promotes neurogenesis in maternal and fetal brains [10, 11]. Moreover, the hPRL transports to the target organs and tissues via the blood circulation to bind to two different types of long or short hPRL receptors (hPRLRs) to activate signal pathways including JaK2 activation, Ras-Raf-MAPK pathway, modulatory pathways, PI3K and downstream pathways, and Stats [12]. However, hGH and hPRL exist multiple structural and functional formats, namely different proteoforms. The proteoform can clarify the function and effect of specific protein in human body and is an intuitive and visual expression of the gene. Therefore, the clarification of hormone proteoforms is necessary to understand its biological roles. Protein proteoforms are primarily derived from alternative splicing, post-translational modifications (PTMs), translocation, redistribution, and spatial conformation alterations [13]. The protein proteoforms can be identified with two-dimensional gel electrophoresis (2DGE) and mass spectrometry (MS).

The isoelectric point (*pI*) and relative mass (*Mr*) are the basic characteristics of a proteoform. 2DGE is depended on different *pI* and *Mr* values to separate proteoforms, which first separates proteoforms by different *pI* values in the isoelectric focusing (IEF) direction, and second separates proteoforms by different *Mr* values in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) direction [14]. 2DGE-based Western blotting coupled with specific antibody can detect a proteoform of a specific protein. Thus, the proteoforms of hGH and hPRL are able to be separated and arrayed with 2DGE. MS is an effect method to characterize protein proteoforms and identify PTMs with an analysis of amino acid sequence and determination of PTM sites [15]. The combination of 2DGE and MS plays an important role in detection, identification, and quantification of proteoforms of hGH and hPRL. The MS methods that were used to study the proteoforms of hGH and hPRL were commonly divided into peptide fingerprint (PMF) and tandem mass spectrometry (MS/MS) analyses. PMF data are commonly obtained with matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF) mass spectromety (MS), and MS/MS data can be obtained from MALDI-TOF-TOF MS or liquid chromatography (LC)-electrospray ionization (ESI)-quadruple-ion trap (Q-IT) MS.

This book chapter will mainly review the hormone proteoforms focusing on hGH and hPRL, the methodology of hormone proteoform study, and future perspective to find effective and potential hormone proteoform biomarkers for in-depth understanding of molecular mechanisms, and individualized and precise diagnosis, therapy, and prognostic assessment of hormone-related diseases.

2. Materials and methods

2.1 Tissues and protein extraction

The human control pituitary tissue samples were post-mortem tissues, and human pituitary adenoma tissue samples were obtained from neurosurgery. The detailed information of those tissue samples were described previously [15, 16]. The collected tissues were immediately frozen in liquid nitrogen, and stored at −80°C until used. The protein extraction of pituitary control and adenoma tissues was performed as described previously [17, 18]. In brief, the contaminated blood in each tissue sample was washed with 0.9% NaCl (3 mL, 5×). The proteins were extracted with protein extraction buffer that consists of 2 mol/L thiourea, 7 mol/L urea, 40 g/L CHAPS, 100 mmol/L dithiothreitol (DTT), 5 mol/L immobilized pH gradient (IPG) buffer pH 3–10 NL, and a trace of bromophenol blue, followed by centrifugation (15,000×*g*, 15 min, 4°C). The supernatant was collected as protein sample, and its protein concentration was determined with a Bio-Rad 2D Quant kit (Bio-Rad) [15].

2.2 2DGE and 2DGE-based Western blot

Each protein sample was first separated by IEF with pH 3–10 NL IPG strips (180 mm × 3 mm × 0.5 mm), under the IEF condition that was a gradient from 0 to 250 V within 1 h (125 Vh), a gradient from 125 to 1000 V within 1 h (500 Vh), a gradient from 1000 to 8000 V within 1 h (4000 Vh), a step-and-hold at 8000 V for 4 h (32,000 Vh), and a step-and-hold at 500 V for 0.5 h (250 Vh) to achieve a total of 36,875 Vh within ~7.5 h [19]. After IEF, proteins were reduced with DTT, and alkylated with iodoacetamide, and then were separated with the 12% SDS-PAGE resolving gel (250 mm × 215 mm × 1.0 mm) in the Tris-glycine-SDS electrophoresis buffer that contained 192 mmol/L glycine, 25 mmol/L Tris-base, and 1 g/L SDS with an electrophoresis condition (250 V, 360 min, 25°C) [20]. The 2DGE-separated proteins were visualized with silver-staining [21].

After 2DGE, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane, blocked with bovine serum albumin (BSA), and incubated with hormone antibodies and secondary antibody. The proteins on the membrane were visualized with 5-bromo-4-chloro-3-indolyl phosphate [15].

The silver-stained 2D gels and 2DE-Western blot images were digitized and analyzed with Discovery Series PDQuest 2D Gel Analysis software [22, 23].

2.3 MS-characterization

2.3.1 MALDI-TOF MS

The tryptic peptide mixture was purified with ZipTipC18, and analyzed with a Perspective Biosystems MALDI-TOF Voyager DE-RP MS (Framingham, MA, USA). The PMF data were obtained to search Swiss-Prot database with PeptIdent software for protein identification. The detailed procedure was described previously [22].

2.3.2 LC-ESI-Q-IT MS

The tryptic peptide mixture was purified with ZipTipC18, and analyzed with LC-ESI-Q-IT MS on an LCQ^{Deca} mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The MS/MS data were obtained to search Swiss-Prot database for protein identification. The detailed procedure was described previously [22].

2.3.3 MALDI-TOF-TOF MS

The tryptic peptide mixture was purified with ZipTipC18, and analyzed with MALDI-TOF-TOF MS on Perspective Biosystems UltraFlex III MALDI-TOF-TOF (Bruker Daltonics). The MS and MS/MS data were obtained to search Swiss-Prot database for protein identification. The detailed procedure was described previously [15].

2.4 Bioinformatics

Many phosphorylation sites of hGH proteoforms were identified with MS/MS, and deamidation was found in many hGH proteoforms. The PTM sites of hPRL in human pituitary adenoma and control tissues, including phosphorylation sites, N-glycosylation sites, and O-glycosylation sites were predicted with NetPhos 3.1 Server (http://www.cbs.dtu.dk/services/NetPhos) [24, 25], NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc) [26], and NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc) [27], respectively.

3. Results and discussion

3.1 MS-identification of open reading frames (ORFs) of hGH and hPRL proteoforms within 2DGE pattern, and confirmed with Western blot

Over 1000 protein spots were found in each pituitary 2D gel (n = 30). MS is an effective method to identify ORFs of a gene across 2DGE map, generally cannot identify proteoforms with the common procedure because MS only detects a partial amino acid sequence but not its complete sequence of a protein. However, each proteoform has its specific *pI* and *Mr*, which can be separated with 2DGE. 2DGE coupled with MS can effectively array and identify proteoforms that are derived from the same gene. Thus, 24 hGH proteoforms (**Figure 1A**) [1] and 6 hPRL proteoforms (**Figure 1B**) [15] were MS-characterized within pituitary 2DGE map.

Some hGH proteoforms and hPRL proteoforms within 2DGE map (**Figure 1**) were also validated with Western immunoblot. For example, four hPRL proteoforms were confirmed with 2DGE-Western blot (**Figure 2**). It demonstrated that 2DGE-MS was an effective and reliable approach to detect and identify proteoforms derived from hGH and hPRL genes. The other hGH proteoforms and hPRL proteoforms were not be validated with immnuoblot, which might be due to no-reactivity of antibody to a specific proteoform.

The hGH proteoforms or hPRL proteoforms in each 2D spot were identified with MS including PMF and MS/MS. For example, the protein in spot 6 in **Figure 1A** was identified as hGH isoform 1 (P01241) with MALDI-TOF PMF data (**Figure 3**). The protein in spot v6 in **Figure 1B** was identified as hPRL (P01236) with MALDI-TOF-TOF PMF data (**Figure 4**), and MS/MS data (**Figure 5**).

Therefore, 2DGE-MS clearly identified 24 hGH proteoforms and 6 hPRL proteoforms with different p*I*-M_r distributions on a 2DGE pattern. Furthermore,

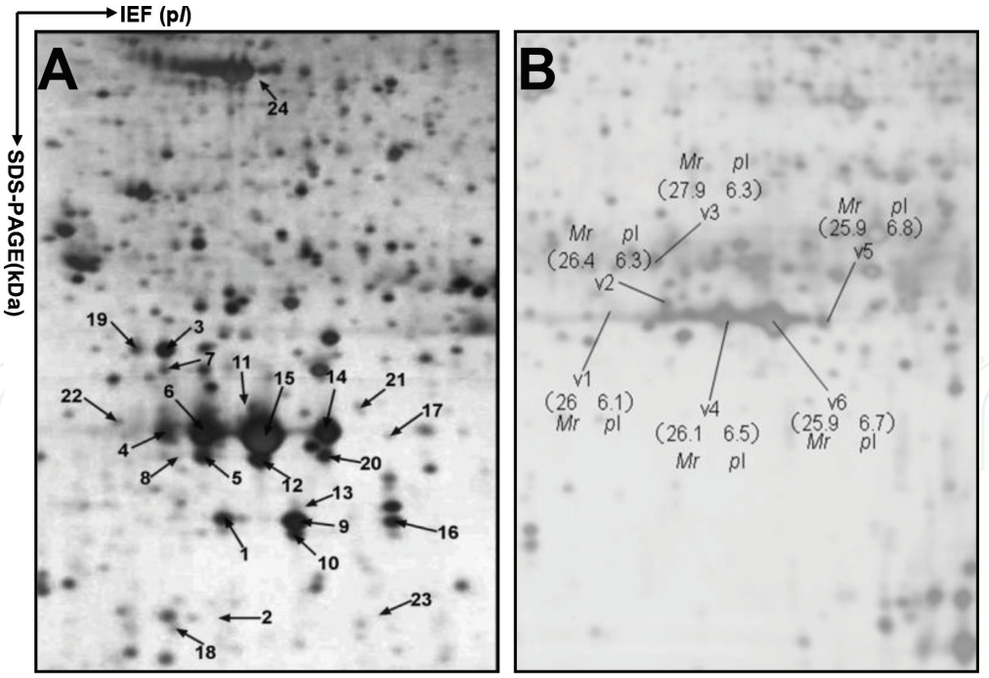

Figure 1.
2DGE patterns of hGH proteoforms (A) and hPRL proteoforms (B) in human pituitaries. The total proteins extracted from pituitary tissues were separated with IPG strip pH 3–10 NL and 12% SDS-PAGE gel, and stained with silver-staining. Modified from Zhan et al. [1], with permission from Wiley-VCH, copyright 2005; and from Qian et al. [15], with permission from Frontiers publisher open access article, copyright 2018.

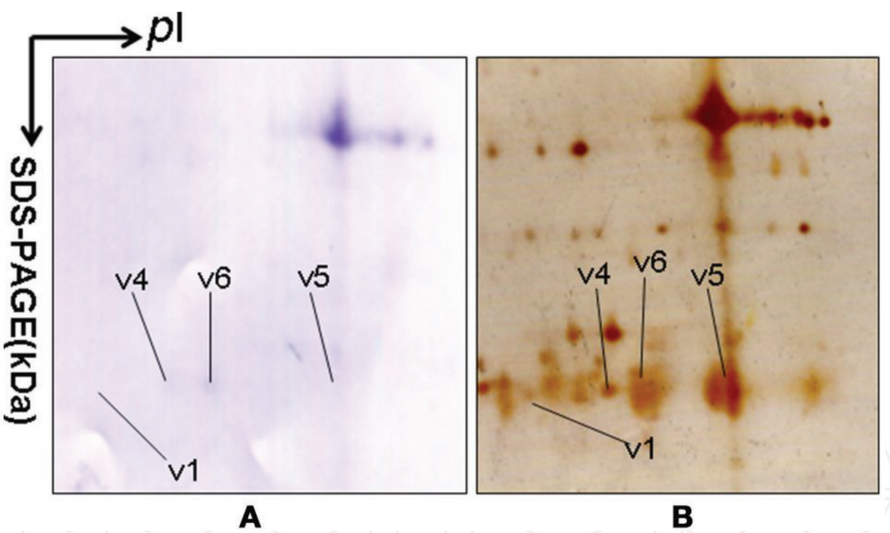

Figure 2.
hPRL proteoforms were validated with 2DGE-Western blot analysis. (A) Western blot image of hPRL proteoforms (rabbit anti-hPRL antibodies + goat anti-rabbit alkaline phosphatase-conjugated IgG). (B) Silver-stained image on a 2D gel after proteins were transferred to a PVDF membrane. Reproduced from Qian et al. [15], with permission from Frontiers publisher open access article, copyright 2018.

one investigated the reasons to form those hormone proteoforms, including signal peptide, splicing variation, and PTMs such as deamination, phosphorylation, and glycosylation.

3.2 MS-determination of signal peptide contained in each hGH or hPRL proteoform in human pituitaries

If signal peptide is contained in the sequence of hGH or hPRL, then it means that hormone is a prohormone (**Figure 6**). It is necessary to determine whether signal

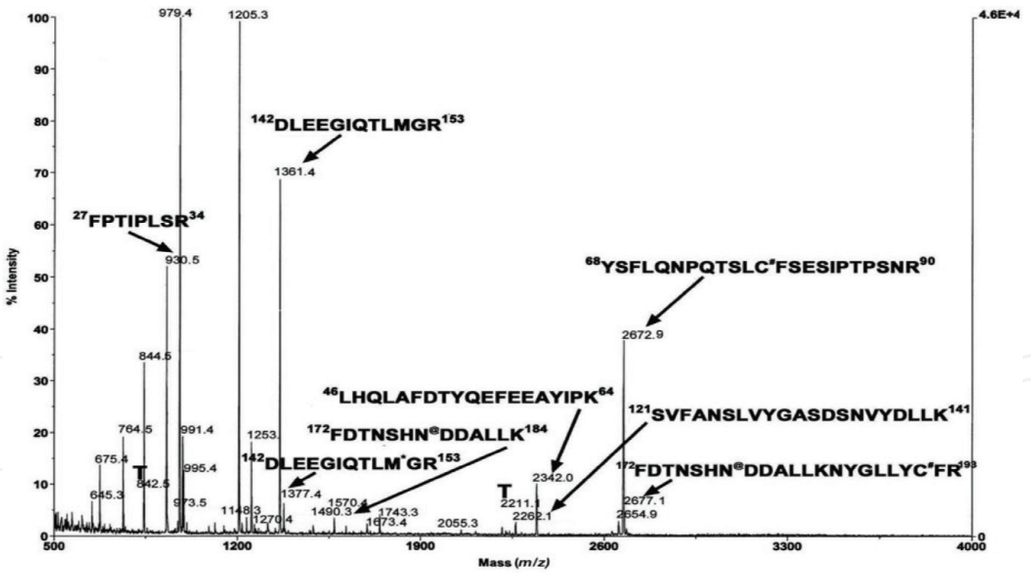

Figure 3.
MALDI-TOF MS spectrum of hGH isoform 1 (spot 6). T = autodigested fragments of trypsin. M = oxidized Met. C# = carbamidomethyl-Cys. N@ = deamidated Asn. Reproduced from Zhan et al. [1], with permission from Wiley-VCH, copyright 2005.*

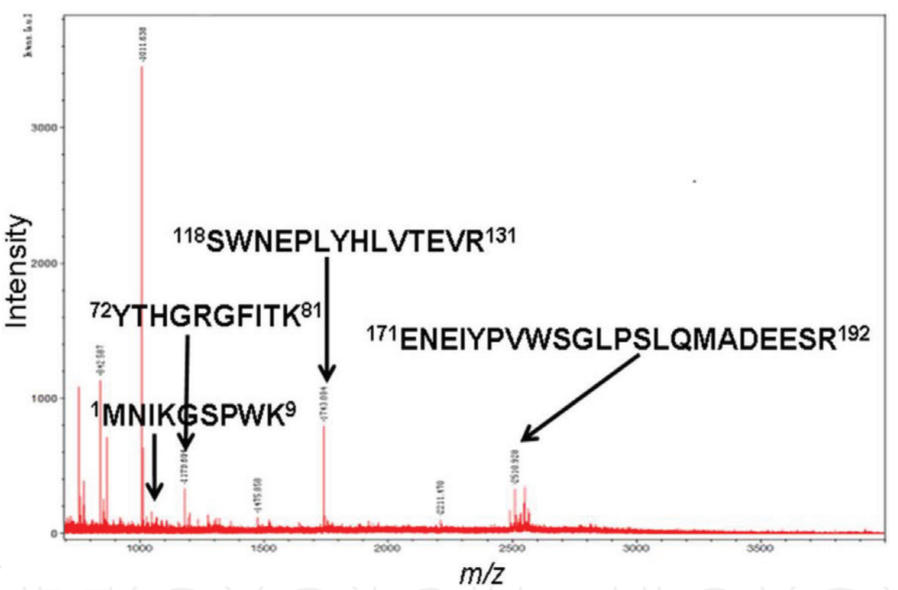

Figure 4.
MALDI-TOT-TOF MS spectrum of hPRL (spot v6). Reproduced from Qian et al. [15], with permission from Frontiers publisher open access article, copyright 2018.

peptide is contained in each hGH proteoform or hPRL proteoform. MS can identify the characteristic tryptic peptide sequences before and after removal of signal peptide in hGH or hPRL (**Table 1**). For 24 hGH proteoforms in human pituitaries, checking all MS data of those 24 hGH proteoforms, the tryptic peptide sequence FPTIPLSR (position 27–34) (**Figure 3**) was detected for each hGH proteoform, but no any characteristic tryptic peptide sequence before removal of signal peptide was found (**Table 1**). It clearly demonstrated that no signal peptide sequence was contained in those 24 hGH proteoforms in the 2DGE pattern (**Figure 1A**). For 6 hPRL proteoforms in human pituitaries, checking all MS data of those 6 hPRL proteoforms, the tryptic peptide sequence MNIKGSPWK (position 1–9) (**Figure 4**) was detected for each hPRL proteoform, but no any characteristic tryptic peptide sequence after removal of signal peptide was found (**Table 1**). It clearly demonstrated that signal peptide sequence was contained in those 6 hPRL proteoforms in the 2DGE pattern (**Figure 1B**).

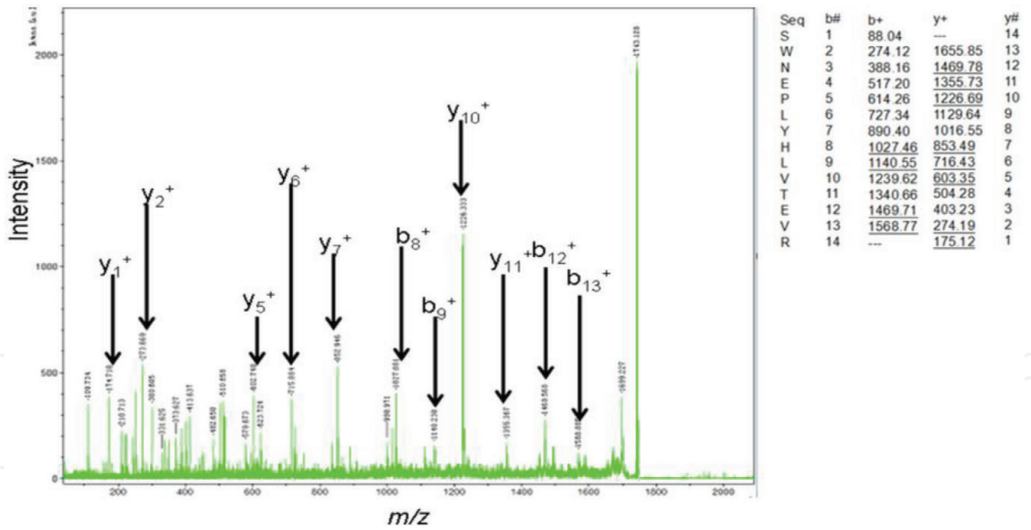

Figure 5.
MALDI-TOF-TOF MS/MS spectrum of the tryptic peptide ^{118}SWNEPLYHLVTEVR^{131} from hPRL in spot v6. Reproduced from Qian et al. [15], with permission from Frontiers publisher open access article, copyright 2018.

hGH	1	11	21	31	41	
1	**MATGSRTSLL**	**LAFGLLCLPW**	**LQEGSA**FPTI	PLSRLFDNAM	LRAHRLHQLA	50
51	FDTYQEF**EEA**	**YIPKEQKYSF**	**LQ**NPQTSLCF	SESIPTPSNR	EETQQKSNLE	100
101	LLRISLLLIQ	**SWLEPVQFLR**	**SVFANSLVYG**	**ASDSNVYDLL**	**KDLEEGIQTL**	150
151	**MGRLEDGSPR**	**TG**QIFKQTYS	KFDTNSHNDD	ALLKNYGLLY	CFRKDMDKVE	200
201	TFLRIVQCRS	VEGSCGF				

hPRL	1	11	21	31	41	
1	**MNIKGSPWKG**	**SLLLLLVSNL**	**LLCQSVAP**LP	ICPGGAARCQ	VTLRDLFDRA	50
51	VVLSHYIHNL	SSEMFSEFDK	RYTHGRGFIT	KAINSCHTSS	LATPEDKEQA	100
101	QQMNQKDFLS	LIVSILRSWN	EPLYHLVTEV	RGMQEAPEAI	LSKAVEIEEQ	150
151	TKRLLEGMEL	IVSQVHPETK	ENEIYPVWSG	LPSLQMADEE	SRLSAYYNLL	200
201	HCLRRDSHKI	DNYLKLLKCR	IIHNNNC			

Figure 6.
The amino acid sequences of hGH and hPRL prohormones. Modified from Zhan et al. [1], with permission from Wiley-VCH, copyright 2005; and from Qian et al. [15], with permission from Frontiers publisher open access article, copyright 2018.

3.3 MS-identification of splicing variants of hGH in human pituitaries

MS is an effective method to identify splicing variants of hGH. hGH splicing variant 2 is derived from deletion of positions 58–72 from hGH, hGH splicing variant 3 is derived from deletion of positions 111-148 from hGH, and hGH splicing variant 4 is derived from deletion of positions 117-162 from hGH (**Figure 6**). The characteristic tryptic peptides for each hGH splicing variant can be theoretically obtained and observed by MS (**Table 2**). The MS data of 24 hGH proteoforms were checked one-by-one. The results showed that hGH splicing variant 2 existed in spot 9, hGH splicing variant 3 existed in spots 2 and 23, and hGH splicing variant 4 existed in spot 13. hGH variant 1 existed in the rest 20 spots.

However, hPRL does not have splicing variants. The *Mr* of hPRL proteoforms was 26.0 kDa for v1, 26.1 kDa for v4, 25.9 kDa for v5, and 25.9 kDa for v6 in 2DGE pattern (**Figure 1**), which was almost equal to the *Mr* (25.9 kDa) of hPRL prohormone. Moreover, the *Mr* of hPRL proteoforms was 26.4 kDa for v2, and 27.9 kDa for v3, which is slightly greater than *Mr* (25.9 kDa) of hPRL prohormone. These data further confirmed that those six hPRL proteoforms were not generated from mature hPRL (position 29–227) but

Hormone	Before and after removal of signal peptide	Position	Characteristic tryptic peptide sequence	Calc. $[M + H]^+$	Observed $[M + H]^+$
hGH (signal peptide sequence 1–26)	Before	1–6	MATGSR	622.3 (MSO: 1; 638.3)	–
		7–34	TSLLLAFGLLCLPWLQEGSAFPTIPLSR	3043.7 3100.7(Cys_CAM: 17)	– –
		1–34	MATGSRTSLLLAFGLLCLPWLQEGSAFPTIPLSR	3646.9 3704.0 (Cys_CAM: 17) 3662.9 (MSO: 1)	– – –
		7–42	TSLLLAFGLLCLPWLQEGSAFPTIPLSRLFDNAMLR	4004.2 4061.2 (Cys_CAM: 17) 4020.1 (MSO: 40)	– – –
	After	27–34	FPTIPLSR	930.5	+
		27–42	FPTIPLSRLFDNAMLR	1891.0 1907.0 (MSO: 40)	± –

Hormone	Before and after removal of signal peptide	Position	Characteristic tryptic peptide sequence	Calc. $[M + H]^+$	Observed $[M + H]^+$
hPRL (signal peptide sequence 1–28)	Before	1–4	MNIK	505.2803	–
		1–9	MNIKGSPWK	1060.5608	+
		1–38	MNIKGSPWKGSLLLLLVSNL LLCQSVAPLPICPGGAAR	3930.1893	–
		5–9	GSPWK	574.2983	–
		5–38	GSPWKGSLLLLLVSNLLLCQ SVAPLPICPGGAAR	3443.9268	–
		10–38	GSLLLLLVSNLLLCQSVAPL PICPGGAAR	2888.6463	–
		10–44	GSLLLLLVSNLLLCQSVAPL PICPGGAARCQVTLR	3589.0154	–
	After	29–38	LPICPGGAAR	954.5189	–
		29–44	LPICPGGAARCQVTLR	1654.8879	–
		29–49	LPICPGGAARCQVTLRDLFD R	2301.1954	–

Modified from Zhan et al. [1], with permission from Wiley-VCH, copyright 2005; and from Qian et al. [15], with permission from Frontiers publisher open access article, copyright 2018. Note: +: this peptide ion was observed in each MS spectrum. –: this peptide ion was not observed by MS. ±: tryptic peptide ion was detected in some but not all PMF data from 24 hGH proteoforms.

Table 1.
Characteristic tryptic peptides before or after removal of signal peptide sequence in hGH or in hPRL.

GH variants	Tryptic peptide that covers the splicing site	Calculated [M + H] + ion (*m/z*)	Observed $[M + H]^+$ ion (*m/z*) in PMF data	hGH proteoforms (Spot No.)
1	^{142}DLEEGIQTLMGR^{153}	1361.7 1377.7 (MSO: 151)	One or two ions were detected to exclude splicing variants 3 and 4 One, two or three ions were detected to exclude splicing variant 2	1; 3–8; 10–12; 14–22; 24
	^{121}SVFANSLVYGASDSNVYDLLK^{141}	2262.1		
	^{46}LHQLA FDTYQEFEEAYIPK^{64}	2342.1		
	^{68}YSF LQNPQTSLCFSESIPTPSNR^{90}	2616.2 2673.2 (Cys-CAM: 79)		
	^{43}AHRLHQLAFDTYQ EFEEAYIPK^{64}	2706.3		
2	LHQLAFDTYQEF^{58}NPQTSLCFSESIPTPSNR	3470.6 3527.7 (Cys-CAM: 64)	— 3527.4	9
	AHRLHQLAFDTYQEF^{58}NPQTSLCFSESIPTPSNR	3834.8 3891.9 (Cys-CAM: 64)	— —	
	LHQLAFDTYQEF^{58}NPQTSLCFSESIPTPSNREETQQK	4213.9 4271.0 (Cys-CAM: 64)	— —	
3	ISLLLIQ^{111}TLMGR	1357.8 1373.8 (MSO: 113)	1357.7 —	2; 23
	ISLLLIQ^{111}TLMGRLEDGSPR	2112.2 2128.2 (MSO: 113)	— —	
	SNLE LLRISLLLIQ^{111}TLMGR	2183.3 2199.3 (MSO: 113)	— —	
4	ISLLLIQSWLEPV^{117}QIFK	2027.2	2027.0	13
	ISLLLIQSWLEPV^{117}QIFKQTYS K	2634.5	—	
	SNLE LLRISLLLIQSWLEPV^{117}QIFK	2852.7	—	

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Table 2.
Determination of the hGH splicing variants.

hGH proteoforms (Spot No.)	Splicing variants	Phosphorylation (p)	Deamidation (d)
1	1/2	pSer176	
2	3; 1/2		
3	1	pSer132	
4	1		
5	1	pSer176;	dAsn178
6	1	pSer77, pSer132, pSer176	dAsn178
7	1		
8	1	pSer176, pSer132	
9	2	pSer176	
10	1	pSer176	
11	1	pSer77, pSer132	
12	1	pSer176; pSer132	
13	4; 3		
14	1	pSer132	
15	1	pSer176, pSer132	
16	1	pSer176	dAsn178
17	1		
18	1	pSer176, pSer132	
19	1		
20	1		
21	1		
22	1		
23	3; 1		
24	1	pSer132	

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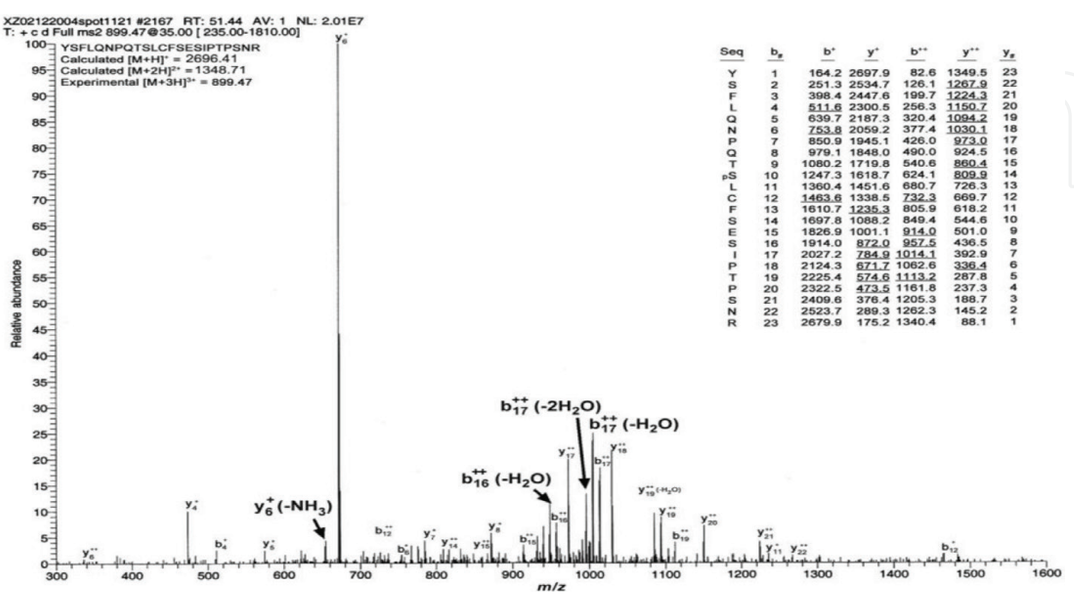
Table 3.
PTMs were identified in hGH proteoforms.



Seq		b	b*	y*	b**	y**	
Y	1		164.2	2697.9	82.6	1349.5	23
S	2		251.3	2534.7	126.1	1267.9	22
F	3		398.4	2447.6	199.7	1224.3	21
L	4		511.6	2300.5	256.3	1150.7	20
Q	5		639.7	2187.3	320.4	1094.2	19
N	6		753.8	2059.2	377.4	1030.1	18
P	7		850.9	1945.1	426.0	973.0	17
Q	8		979.1	1848.0	490.0	924.5	16
T	9		1080.2	1719.8	540.6	860.4	15
pS	10		1247.3	1618.7	624.1	809.9	14
L	11		1360.4	1451.6	680.7	726.3	13
C	12		1463.6	1338.5	732.3	669.7	12
F	13		1610.7	1235.3	805.9	618.2	11
S	14		1697.8	1088.2	849.4	544.6	10
E	15		1826.9	1001.1	914.0	501.0	9
S	16		1914.0	872.0	957.5	436.5	8
I	17		2027.2	784.9	1014.1	392.9	7
P	18		2124.3	671.7	1062.6	336.4	6
T	19		2225.4	574.6	1113.2	287.8	5
P	20		2322.5	473.5	1161.8	237.3	4
S	21		2409.6	376.4	1205.3	188.7	3
N	22		2523.7	289.3	1262.3	145.2	2
R	23		2679.9	175.2	1340.4	88.1	1

Figure 7.
MS^2 spectrum of a phosphopeptide ($[M + 3H]^{3+}$: m/z = 899.47; retention time = 51.44 min; scan number 2167) derived from hGH proteoform 6 (Spot 6). Reproduced from Zhan et al. [1], with permission from Wiley-VCH, copyright 2005.

Sequence	#	x	Context	Score*	Kinase	Answer
Sequence	6	S	NIKGSPWKG	0.779	unsp	YES
Sequence	11	S	PWKGSLLLL	0.848	PKA	YES
Sequence	18	S	LLLVSNLLL	0.523	cdc2	YES
Sequence	42	T	RCQVTLRDL	0.891	unsp	YES
Sequence	61	S	IHNLSSEMF	0.718	unsp	YES
Sequence	62	S	HNLSSEMFS	0.553	unsp	YES
Sequence	66	S	SEMFSEFDK	0.991	unsp	YES
Sequence	72	Y	FDKRYTHGR	0.503	INSR	YES
Sequence	73	T	DKRYTHGRG	0.557	unsp	YES
Sequence	90	S	CHTSSLATP	0.585	DNAPK	YES
Sequence	93	T	SSLATPEDK	0.737	unsp	YES
Sequence	110	S	KDFLSLIVS	0.507	PKA	YES
Sequence	118	S	SILRSWNEP	0.749	unsp	YES
Sequence	124	Y	NEPLYHLVT	0.956	unsp	YES
Sequence	142	S	EAILSKAVE	0.517	CKII	YES
Sequence	151	T	IEEQTKRLL	0.983	unsp	YES
Sequence	163	S	ELIVSQVHP	0.623	ATM	YES
Sequence	169	T	VHTEPKENE	0.541	CKII	YES
Sequence	175	Y	ENEIYPVWS	0.804	unsp	YES
Sequence	191	S	ADEESRLSA	0.576	cdc2	YES
Sequence	194	S	ESRLSAYYN	0.982	unsp	YES
Sequence	207	S	LRRDSHKID	0.993	unsp	YES

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**Score > 0.5 means a statistically significant result.*

Table 4.
Prediction of phosphorylation sites in hPRL prohormone (positions 1–227) with NetPhos server with a score more than 0.5.

Seq name	Position	Potential	Jury agreement	N-Glyc result	
Sequence	2 NIKG	0.7530	**(9/9)**	+++	
Sequence	19 NLLL	0.7151	**(9/9)**	++	
Sequence	59 NLSS	0.7380	**(9/9)**	++	SEQUON ASN-XAA-SER/THR.
Sequence	84 NSCH	0.7312	(8/9)	+	
Sequence	104 NQKD	0.6020	(7/9)	+	
Sequence	120 NEPL	0.6051	(6/9)	+	
Sequence	172 NEIY	0.5346	(5/9)	+	
Sequence	198 NLLH	0.5642	(5/9)	+	
Sequence	212 NYLK	0.6726	(8/9)	+	
Sequence	224 NNNC	0.5146	(5/9)	+	
Sequence	225 NNC-	0.3576	(8/9)	—	
Sequence	226 NC--	0.3351	**(9/9)**	—	

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Note: Asparagines predicted to be N-glycosylated are highlighted in bold font.

Table 5.
Prediction of N-glycosylation sites in hPRL prohormone (positions 1–227) with NetNGlyc 1.0 server with score more than 0.5.

from hPRL prohormone (positions 1–227) in human pituitary tissues. These data coupled with MS data clearly demonstrated six hPRL proteoforms were not derived from splicing.

3.4 MS/MS-identification of PTMs in hormone proteoforms

MS/MS was an effective method to identify the PTMs and their modification sites of hGH and hPRL to clarify the reasons of formation of hGH proteoforms and hPRL proteoforms. MS/MS identified phosphorylation at sites Ser-77, Ser-132, and Ser-176 in many hGH proteoforms (**Table 3**). A representative MS/MS spectrum was shown to determine phosphorylation site Ser-77 in hGH proteoform 6 (Spot 6) (**Figure 7**). Also, deamidation was found in many hGH proteoforms. In addition, there would be other PTMs in hGH proteoforms that need to be further characterized.

#Seq name	Source	Feature	Start	End	Score	Strand	Frame	Comment
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	25	25	0.134588	.	.	
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	42	42	0.190888	.	.	
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	54	54	0.194926	.	.	
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	66	66	0.176466	.	.	
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	73	73	0.111052	.	.	
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	80	80	**0.613645**	.	.	#POSITIVE
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	85	85	**0.618483**	.	.	#POSITIVE
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	88	88	**0.602886**	.	.	#POSITIVE
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	89	89	**0.717093**	.	.	#POSITIVE
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	90	90	**0.928857**	.	.	#POSITIVE
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	93	93	**0.778272**	.	.	#POSITIVE
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	128	128	0.181904	.	.	
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	151	151	0.11243	.	.	
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	163	163	0.424529	.	.	
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	169	169	0.122664	.	.	
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	179	179	0.380532	.	.	
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	183	183	0.309982	.	.	
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	191	191	0.1589	.	.	
SEQUENCE	netOGlyc -4.0.0.13	CARBOHYD	194	194	0.249957	.	.	

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Note: The bold value means a statistically significantly positive result.

Table 6.
Prediction of O-glycosylation sites in hPRL prohormone (position 1–227) with NetOGlyc 4.0 server with score more than 0.5.

For hPRL proteoforms, bioinformatics including NetPhos 3.1 Server (http://www.cbs.dtu.dk/services/NetPhos) [24, 25] predicted 14 pS sites, 5 pT sites, and 3 pY sites in the hPRL (**Table 4**), NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc) [26] predicted ten significantly N-glycosylated sites (**Table 5**), and NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc) [27] predicted six significantly O-glycosylated sites in the hPRL (**Table 6**) in human pituitaries. These predicted PTM sites in hPRL proteoforms provided clues and needed to be confirmed with MS/MS in future studies.

4. Conclusions

hGH and hPRL are two important hormones in human endocrine systems, which are synthesized in the pituitary gland and secreted into the circulation system. Clarification of hGH proteoforms and hPRL proteoforms in human pituitary is essential step to elucidate their biological functions. Alternative splicing and PTMs are two important factors to cause proteoforms. 2DGE effectively presented 24 hGH proteoforms and 6 hPRL proteoforms with different p*I*-M_r distributions in 2DGE pattern of pituitary tissue proteome. MS/MS effectively identified their splicing variants and PTMs: (i) 24 hGH proteoforms in pituitary removed their signal peptide, whereas 6 hPRL proteoforms in human pituitary did not remove their signal peptide. (ii) 24 hGH proteoforms in human pituitary are derived from 4 types of alternative splicing variants, whereas 6 hPRL proteoforms do not exist any alternative splicing variants. (iii) PTMs pSer-77, pSer-132, and pSer-176 were identified in some of 24 hGH proteoforms, whereas although no PTMs were identified in hPRL proteoforms with MS/MS. However, phosphorylation, N-glycosylation, and O-glycosylation have been predicted with bioinformatics in hPRL proteoforms. Deamidation was presented in both hGH proteoforms and hPRL proteoforms. Therefore, 2DGE coupled with MS plays crucial roles in detection, identification, and quantification of hormone (hGH and hPRL) proteoforms, which benefits insight into the molecular mechanisms and discovery of effective biomarkers of hormone-related diseases.

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Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations.

Author's contributions

X.Z. conceived the concept, designed the book chapter, wrote and critically revised the book chapter, coordinated and was responsible for the correspondence

work and financial support. Z.T. participated in analysis of references and wrote partial manuscript.

Acronyms and abbreviations

BSA	bovine serum albumin
DTT	dithiothreitol
ESI	electrospray ionization
GHRH	growth hormone-releasing hormone
hGH	human growth hormone
hPRL	human prolactin
hPRLRs	hPRL receptors
IEF	isoelectric focusing
IPG	immobilized pH gradient
IGF-1	insulin-like growth factor-1
JAKs	janus-activating tyrosine kinases
LC	liquid chromatography
MALDI	matrix-assisted laser desorption/ionization
M_r	relative mass
MS	mass spectrometry
MS/MS	tandem mass spectrometry
ORFs	open reading frames
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
PMF	peptide mass fingerprint
PTM	post-translational modification
PVDF	polyvinylidene fluoride
Q-IT	quadruple-ion trap
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TOF	time-of-flight
2DGE	two-dimensional gel electrophoresis

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