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CENTRIFUGAL ULTRAFILTRATION AS TOOL FOR LOW ABUNDANCE PROTEINS ISOLATION

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Abstract
Because of the large amount of albumin and others "high abundance proteins" (HAP), the "dynamic range" and heterogeneity of proteins, the separation, isolation, characterization and quantifying of "low abundance proteins" LAP from serum, most of them important diagnostically marchers, is technically difficult.
The aim this study is to test a simple and cheap method to reduce the serum protein complexity in order to identify and quantify LAP, particularly growth hormone. Ultrafiltration was used as preparative method to remove HAP from serum and enrich the sample in LAP. Denaturation seems to be essential before centrifugal ultrafiltration (Georgiou, 2001/ Tirumalai, 2003/ Harper, 2004). Filtration was performed under denaturing conditions and recovery of hGH calculated. The higher recovery rate (46%) was attempted when the samples was denatured before centrifugal ultrafiltration in buffer containing RapiGest, compared with denaturation in SDS (6,2% recovery).The results are helpful for procedures of quantitative determination of LAP e.g. hGH from serum.
Keywords: ultrafiltration, denaturation, recovery, low abundance protein, quantify
Abbreviations: LAP = „low-abundance" Proteine ; HAP = „high-abundance" Proteine ; LMW = „Low Molecular Weight" ; 2D-LC = two-dimensional Chromatographie; Iodacetamide = IAA; DTT = Dithiothreitol; AcCN- "acetonitrile". RT= "retention time", SDS= "sodium dodecylsulphate"

INTRODUCTION

The current methods of proteomics are not forward as the content of serum proteins to catalogue. However only a limited publication refers to identification of "low-abundance" proteins and less to quantification.

Therapy of various diseases needs an exactly diagnosis. Dysfunction of growth for example (acromegaly, deficiency in growth hormone) arise the necessity to establish a correct diagnostic. By children the exact determination of limit between health and pathologic is important in decision to begin a treatment or not, taking in account that therapy is expensive. Determination of hGH is dependent on the method used. The variability of results is very high especially when methods based on antibody are used. One way to reach an exact value of determination of hGH and other low abundance proteins is to elaborate a standard method of quantification. That means first the identification of the low abundance proteins needs consequently the separation of LAP from serum.

For identification of LAP from serum were performed different analytical methods as fractionation, separation and concentration.

The main protein components of serum as quantity are: albumin (55%), immunoglobulin (60-90%), transferrin, haptoglobin and lipoproteins. Excepting the "high abundance proteins" (HAP), there are in serum "low abundance proteins" (LAP) used in clinical chemistry as relevant diagnostic marchers (proteins that modify their concentration and expression depending on pathology). Generally, about 22 proteins account 99% from serum proteins concentration and the rest of 1% are LAP, diagnostic marchers (Tirumalai, 2004). Precise determination of LAP is essential for clinical goals. Quantitative difference between HAP (mg/ml) and LAP (lower as 10 ng/ml) on a large scale logarithmic cover 10 order (Anderson, 2002).

In a schematically draw of 70 serum proteins, albumin is the most abundant (35-55 mg/ml, 35-40x 10^9 pg/ml), indicator of hepatic pathology and malnutrition. The other end is occupied by LAP, e.g. Interleukin 6 (0-5 pg/ml), an indicator for infections and inflammations (Sacher, 2000), and growth hormone (hGH) (16 ng/ml), an indicator for growth diseases (Boguszewski, 2003), dopping (Sonksen, 2001), diabetes, hypophyse cancer.

Because of the large amount of albumin and others HAP, the "dynamic range" and heterogeneity of proteins, the separation, isolation and characterization and quantifying of LAP from serum is technically difficult. This is for why HAP must be removed from serum in order to make possible the identification and quantify of LAP.

Different analytical methods: fractionation, isolation, separation concentration were used for identification and characterization of LAP from serum. The major strategy was the multidimensional separation of intact proteins or peptides from mixtures after enzyme digestion in order to characterize and catalogs the new serum proteins.

A simple method of separation, centrifugal ultrafiltration was checked for the performance to recover the low abundance proteins from serum after separation.

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MATERIALS AND METHODS

Materials

Amicon Ultra-15 Centrifugal Filters Devices, MWCO 50000 (Cat. No. UFC 905008, Firma Millipore, Ultracel™ Low Binding regenerated Cellulose); human growth hormone from Hypophyse extract and Somatotropin (STH) from human pituitary was purchased by Sigma; Tris, ammonium bicarbonate from Sigma, *RapiGest* SF from Waters;

Methods

1. Ultrafiltration.

Ultrafiltration were performed on Amicon Ultra-15 Centrifugal Filters Devices, MWCO 50000, under denaturation. Ultrafiltration on Amicon Ultra-15 (cut-off 50K) were performed under denaturing. One sample remain not filtered (control sample).

hGH (human growth hormone from Hypophyse) (2 nmol) was solubulized in different buffers before centrifugation as follow:

buffer A: 25 mM ammonium bicarbonate, pH 8.2

buffer B: 25 mM ammonium bicarbonate, pH 8.2, 20% Acetonitril

buffer C: 25 mM ammonium bicarbonate, pH 7,8, 7,5 M urea

buffer D: 25 mM ammonium bicarbonate, pH 8.2; 0.01% SDS

bufferE: 53,6 mM Tris, pH 8 + 5,36 mM DTT + 0,214% RapiGest

buffer F: 25 mM ammonium bicarbonate, pH 8.2; 20% Acetonitril; 5,3 mM DTT

buffer G: 25 mM ammonium bicarbonate, pH 7,8; 7,5 M urea; 5,3 mM DTT

buffer H: 25 mM ammonium bicarbonate, pH 8.2; 0.01% SDS; 5,3 mM DTT

buffer I: 25 mM ammonium bicarbonate, pH 8.2; 5,3 mM DTT

2. Sample preparation

Somatotropin / human Growth Hormone from pituitaries/ Sigma (2 nmol= 40 μg) was solubilized each in:

Probe A: 1 ml Buffer A + 2 ml distilled water; Probe B: 1 ml Buffer B + 2 ml distilled water; Probe C: 1 ml Buffer C + 2 ml distilled water; Probe D: 1 ml Buffer D + 2 ml distilled water; Probe E: 0,1 ml Buffer E + 2,9 ml distilled water; Probe F: 3 ml Buffer F; Probe G: 3 ml Buffer G; Probe H: 3 ml Buffer H; Samples E,F, H were boiled 5 minutes at 100 °C before ultrafiltration.

3. Ultrafiltration on Amicon Ultra Centrifugal Filter Devices MWCO 50000

Ultrafiltration wasperformed as recomended by propducer 4000 rpm, 40 minutes. The filers were each wahed with water 3 ml and centrifuge again to avoid the loosing of hGH material.

After solvent deshidratation, the fliltered samples were reloaded in the same buffer (500 μl buffer I) for comparision of filtration efficiency.

4. Sample preparation for mass spectrometry

4.1. Denaturation. Samples A,B,D,E,F,H, were supposed to denaturation 5 minute 100 °C. Samples C,G (with urea) remain at room temperature.

4.2. Reducing. Samples A-H were supposed to reduction 1 hour 56 °C. Cooling at room temperature.

4.3. Alkylation with iodacetamide (IAA) (Sigma, Cat. No. A-3221). Alkylation's was performed by adding to each sample (500 μl) of 15 μl solution 0,5 mol/L IAA to give a final concentration of 15 mM IAA, 60 minutes at room temperature and dark.

4.4. Trypsin digestion. Trypsin (Sigma) (4 μl) were added to each samples 18 hours ratio enzyme: protein 1: 50. A second digestion was started for others 25 hours by adding 4 μl trypsin again. After 41 h digestion was added again the same amount tripsin 1: 50 ratio. After 53 h digestion was stopped with 3 μl HCl conc. at each samples. All the samples were freezed at – 20 °C.

5. LC-MS analysis.

Each samples was solubilized in 1 ml Solvent A (H_2O + HCOOH) + 10% solvent B (AcCN + 0,1% HCOOH).

Experimental conditions: Column Phenomenex Jupiter 5 μ C18 300 A, 150x 2 mm. Solvents A and B. Gradient: 10% B 60 minutes, 30 % B 70 minutes, 50% B 70 Minutes, 100 % B 85 minutes, 10% B 90 minutes. Flow 0,4 ml/minute. SIM modus, scan m/z 603,3 [159-167] (+2) common fragment 20K and 22k hGH, 892,2 [42-64] (+3), 22 K characteristic. Spray: 13 l/min. Chamber: gas temperature 350 °C, Vcap positive 6000, negative 3500. UV 280 nm.

RESULTS AND DISCUSSIONS

Human growth hormone is synthesized by hypophyse. hGH is a mixture of variants, forms, posttranslational modification and fragments. each form is a separately hormone with proper physiological characteristic. hGh belongs to one of the most various heterogeneous class of peptide hormones. hGHs consists of isoforms (proteins with different primary structure) and variants (molecular forms derived from the same primary structure) (Baumann, 1991). The most spreeded variant of hGH is 22 K hGH (Fig. 1). 20K is the most spread isoform of "non-22K" hGH (Fig. 2). The ratio 20K/22K in blood is approximately constant under normally conditions.

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FPTIPLSRLF DNAMLRAHRL HQLAFDTYQE FEEAYIPKEQ KYSFLQNPQT SLCFSESIPT PSNREETQQK SNLQLLRISL LLIQSWLEPV QFLRSVFANS LVYGASNSDV YDLLKDLEEG IQTLMGRLED GSPRTGQIFK QTYSKFDTNS HNDDALLKNY GLLYCFRKDM DKVETFLRIV QCRSVEGSCG F

Fig. 1. Primary structure of 22K hGH (Bauman).

FPTIPLSRLF DNAMLRAHRL HQLAFDTYQE FEEAYIPKEQ KYSFLQNPQT SLCFSESIPT PSNREETQQK SNLQLLRISLLLIQSWLEPV QFLRSVFANS LVYGASNSDV YDLLKDLEEG IQTLMGRLED GSPRTGQIFK QTYSKFDTNS HNDDALLKNY GLLYCFRKDM DKVETFLRIV QCRSVEGSCG F

Fig. 2. Primary structure of 20K hGH (Bauman).

By theoretical digestion with trypsin, and modification with IAA (http://prospector.ucsf.edu/prospector) two cys-containing petide were chosed for measurements m/z 603,3 [159-167] (+2) common peptide for 20K and 22K hGH, and 892,2 [42-64] (+3), characteristic peptide for 22 K hGH. The common peptides carbamidomethyl- derived (with IAA) on cystein, has the following sequence: (K)NYGLLYCFR(K) (Table 1).

Table 1. MS-Digest Search Results for hGH (ProteinProspector)

m/z(mi)	m/z(av)	Start-End	Missed Cleavages	Database Sequence
1205.5777	1206.4178	159-167	0	(K)NYGLLYCFR (K)
402.5311^{+3}	402.8113^{+3}	159-167	0	(K)NYGLLYCFR (K)
603.2928^{+2}	603.7129^{+2}	159-167	0	(K)NYGLLYCFR (K)

All measurements were performed in SIM (single ion monitoring) modus, for characteristic peptide of hGH 22K (m/z 892,2, (+3) sequence [42-64]) and one common peptide of 20K and 22K hGH (m/z 606,3 (+2), [159-167]).

The results are summarized on the Table 2.

Sample	Retention time (min)	pmol injected	m/z	A (absorbance)	Recovery %
E (Rapigest)	38,8	8	603,3	38593,4	51%
	44,5		892,2	27735,7	46,20%
A (control)	39	8	603,3	75309,5	
	44,3		892,2	59940,5	
D (SDS)	38,5	8	603,3	8891,73	11,80%
	41,9		892,2	3742,3	6,20%
B (20% AcCN)		8	603,3	No signal	
			892,2	No signal	
F (20% AcCN + DTT)		8	603,3	No signal	
			892,2	No signal	
H (SDS + DTT)		8	603,3	No signal	
			892,2	No signal	

Table 2. Recovery of hGH after centrifugal filtration on Amicon Ultra-15
Centrifugal Filters Devices, MWCO 50000

Denaturation of proteins before centrifugal filtration was reported as successfully under denaturing conditions, similar methods were used in this experiment. There were 2 steps of denaturation before ultrafiltration. Samples B and F were solubilized in Buffer (buffer B: 25 mM ammonium bicarbonate) supplemented with 20% acetonitrile. Sample F contain supplementary 5 mM DDT and was again supposed to denaturation by boiling at 100 °C in order to reduce S-S bridges. After centrifugal filtration, all the samples were solubilized in buffer I (25 mM ammonium bicarbonate + 5 mM DTT). However, no cystein peptide was identified. Sample H was solubilized

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in buffer H (25 mM ammonium bicarbonate + 5 mM DDT + 0,01% SDS) and then denaturated. As in case of samples B and F, no cystein peptide was identified. In contrast, sample E solubilized in buffer E (53,6 mM Tris, pH 8 + 5,36 mM DTT + 0,214% RapiGest), and sampled (solubilized in buffer D: 25 mM ammonium bicarbonate, pH 8.2; 0.01% SDS) cytein peptides were identified. Is to mention that all peptides choused to be measured in SIM modus contain cystein.

The samples A,B,D,E,F,H were identically manufactured after centrifugal filtration with organic solvents as acetonitrile (samples B,F) or boiled (B,F,H). No cystein containing peptides were identified.

It is difficult to suppose that there is missing cleavages sites (trypsin digestion over 53 hour) or reduction and alkylation did not work. In contrast the samples containing detergents RapiGest, SDS contain also the Cys-peptides, that could be identified and measured.

Unlike other commonly used denaturants, such as SDS or urea, *RapiGest* SF does not modify peptides or suppress protease activity. It is compatible with enzymes such as Trypsin, RapiGest SF improves efficiency of digestion, no adducts or protein modifications observed and is compatible with HPLC and MS analysis. RapiGest SF is an acid-cleavable anionic detergent marketed by Waters Corporation. The higher recovery rate (46%) was attemped when the samples was denatured before centrifugal ultrafiltration in buffer containing Rapigest, compared with denaturation in SDS (6,2% recovery).

For quantitation of C-reactive protein (CRP) from serum are reported in literature different results depending on the standard peptide used for quantitation and separation methods. Similar centrifugal filter have a recovery of 66% CRP from serum when radioactive labelled peptide was added as "spike" into a serum samples. Depending on selected standard recovery was 66%, 24%, 28%, respectively 0,2%. One reason of the differences should be an incomplete enzyme digestion of proteins (Kuhn, 2004).

Denaturation seems to be essential before centrifugal ultrafiltration. Ultrafiltration was used for separation of HAP and enrichment of LAP form serum (Georgiou, 2001/ Tirumalai, 2003/ Harper, 2004). Georgiou used ultrafiltration without denaturation on Centrex UF-0.5 Filter 30K cut off (Schleicher & Schuell, Dassel, Germany) for filtration of 500 µl serum. The separation was unsuccessfully because protein higher as 30 K were founded in filtrate (fraction <30K) and vice versa. Under this experimental conditions, could not identified more protein over SDS-PAGE as from entire plasma.

The same technique centrifugal ultrafiltration on Centriplus centrifugal filters (MWCO 30K, Millipore) under denaturation was used (Tirumalai, 2003). As knows, albumin (aprox. 50% serumsproteins) is one transporter in blood for hormones, cytokines and lipoproteins. Affinity separation on Cibacron Blue give a high leakage of" low Molecular Weight" (LMW) Proteine bounded on albumin (Burtis, 2001)

Denaturation of serum (20% Acetonitryl oder 5M urea) releases components on albumin bounded, some of them proteins, so that no losing are registered. Ultrafiltration on Centriplus centrifugal filters (MWCO 30K, Firma Millipore) was used under denaturation for separation of serum components. The recovery of LMW Proteins was 15-20% and a high number (341) of proteins could be identified, inclusive peptide hormones.

Analysis of literature data shows a different number of identified proteins from serum depended on separation method (Table 3).

Number identified proteins	year	Methode
60	Huges, 1992	PAGE/MS
325	Pieper, 2003	IASC/ Chromatography/ MS
341	Radhakrishna, 2003	Ultrafiltration/MS
405	Rose, 2004	IASC/GF/CEX/ RP
490	Adkins, 2002	IASC/IEX/MS
1444	Chan, 2004	IEF/IEX/RP/MS
4000	PNNL, Wang 2005	FTICR

Table 3. Number of proteins identified from serum depends on separation methods.

IASC-immunoaffinity subtraction chromatography; IEX-ion exchange chromatography; RP-reverse phase chromatography; IEF-isoelectric focusing; CEX-cation exchange chromatography; MS-mass spectrometry; PAGE-poliacrylamide electrophoresis; GF-gel filtration; FTICR-Fourier transform ion cyclotron resonance.

Different methods (referred to plasma denaturation and dilution) and parameters of centrifugation (speed) lead to various results by separation on the same membrane filter (Harper).

Centrifugal Device Filter Centricon, cutt off 3K were used to desalify proteins or removal of lipids from serum before electrophoresis. The recovery was 92% (Joo, 2003).

The number of identified protein from serum varies with separation method. A number of 262 LAP in serum were identified using a methods that include ultrafiltration (Harper, 2004). The author considers that more protein could be identified after improving the multidimensional chromatography step.

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CONCLUSIONS

Removal of high abundance proteins from serum was realized by centrifugal ultrafiltration.

Denaturation of proteins before centrifugation has at least two reasons. The interactions protein-protein could be cleaved under denaturing conditions. Moreover the insoluble components of serum that could clog the membrane filter could be removed by a supplementary centrifugation on device filter.

Ultrafiltration was performed under different denaturing environment.

1. denaturation with organic solvents (20% Acetonitrile) and high temperature (100 °C) leads to totally losing of the sample after centrifugal ultrafiltration, possible because of protein denaturation on filter.
2. In company of detergents (RapiGest, SDS) recovery of HAP is relatively high (46% in case of 22K hGH characteristic peptide.
3. Centrifugal Filter devices Amicon Ultra 15 MWCO 5000 can be used for removal of high abundance protein from serum but with high lost of low abundance proteins.
4. the lowest lost was by denaturing with RapiGest. The higher recovery rate (46%) was attempted when the samples was denatured before centrifugal ultrafiltration in buffer containing RapiGest, compared with denaturation in SDS (6,2% recovery).

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