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A mass spectrometry based method and a software tool to assess degradation status of serum samples to be used in proteomics for biomarker discovery

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ABSTRACT

Integrity of serum samples is a major concern for biomarker discovery in many fields, including oncology. A gradual degradation of the fibrinopeptide A (fpA) as a result of the preservation process was already observed. In this work, we investigated this process to define a method for the assessment of sera integrity based on the total amount of fpA and its degradation pattern.

SeraDeg software was developed. It identifies fpA fragments in spectra and extracts their abundances. The SeraDeg Quality Score (SDQS) is finally determined for each spectrum depending on its total fpA abundance and on the ratio of its higher mass versus lower mass fragments abundances.

Spectra were evaluated for the total amount of fpA and the percent contribution of its fragments. The total fpA abundance is significantly lower in cryo-preserved samples than in fresh sera. Moreover, fresh sera present higher percentages of the most intact forms of fpA. Four relevant fpA fragments were identified.

We developed and made available on-line SeraDeg, a software able to screen serum spectra, compute fpA related parameters and assign quality scores. SeraDeg was validated using spectra from a set of 250 samples.

Biological significance: The susceptibility of fpA to degradation suggests its use as quality indicator of cryo-preserved serum samples. SeraDeg can support the adoption of this method which may especially be useful in differential sero-proteomics for wide-range biomarker discovery. Moreover, it discloses the doors to a critical re-evaluation of previous experiments in light of the quality of serum samples as assessed by fpA degradation analysis.

1. Introduction

Biological samples which have undergone long term storage at − 80 °C are often used in retrospective translational and clinical studies aimed to the identification of potential biomarkers. In the case of rare diseases or during the assessment of the long-term effects of a certain risk condition, samples have been collected over several years or even decades. Therefore, in order to obtain reliable results, it is essential ensuring that the biological source material was properly stored over the years. Beside the preservation conditions, also the sample handling before freezing may greatly affect the quality of the samples. During proteomic analysis of colon cancer tissue performed by SELDI-ToF, Spruessel and co-workers showed that the intensity of 30% of protein peaks changed significantly (> 2-fold) within the first 30 min following surgical excision [1]. Due to these well-known issues, one of the potential risks in a proteomic approach is then that data obtained from samples with different preservation time and conditions are compared. For example, pathological serum samples collected over several years and cryo-preserved in biobanks could typically be compared with control samples recently obtained from healthy donors. In this case, the statistical analysis aimed to the identification of differences between the two groups could identify as biomarkers of pathological condition signals whose difference could actually be attributable to the degradation process of the pathological samples.

Rai and colleagues published in 2005 a detailed study on the most important pre-analytical variables able to alter the analysis of blood-derived samples [2]. They included patient conditions, venepuncture details, collection and storage characteristics.

Based on the information acquired from several groups worldwide, an attempt to standardize the whole procedure was made by the UK Biobank [3]. This effort resulted in the development of the UK Biobank sample handling and storage protocol.

However, the proposed standardized approach is useful for the design of new prospective studies, but it is ineffective in order to assess the integrity of serum samples already stored in biobanks.

Gislefoss and co-workers [4] studied the effects of pre-analytical

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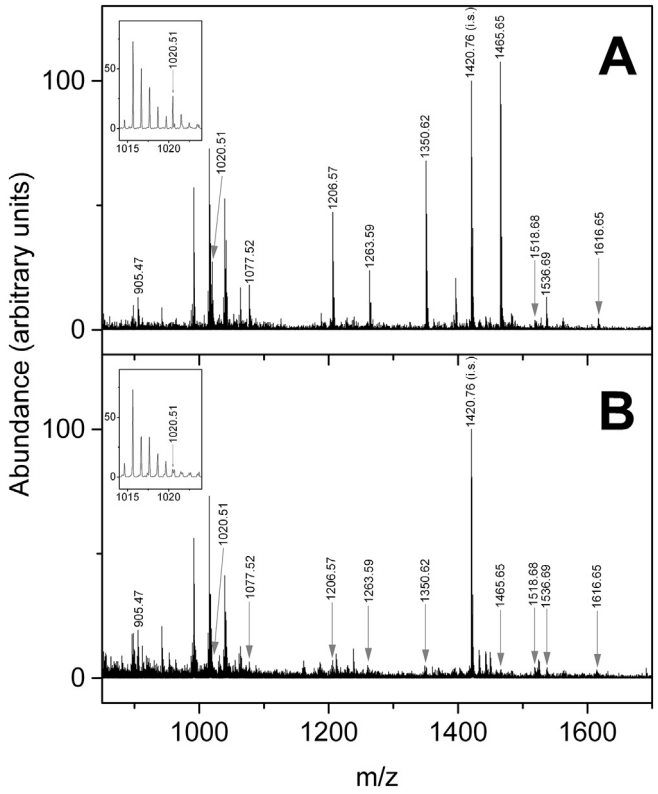

Fig. 1. MALDI-ToF spectra from fresh (panel A) and poorly preserved (panel B) samples. All fpA derived fragments and the internal standard (i.s., at *m/z* 1420.76) are labelled with mono-isotopic *m/z* value. The intervals around the fragment at 1020.55 have been zoomed.
All the fpA fragments in the poorly preserved samples are decreased, but the 905.47 one.

sample handling and storage conditions by testing the stability of different components of serum samples, such as electrolytes, minerals and water soluble molecules. Unfortunately, from these data one could hardly get any information about the degradation of the proteic component of the sample, which can also be affected by endogenous proteolytic activities.

The term peptidome refers to the low molecular weight region of the protein fraction contained in a given biological sample. The peptidomic profile of a fresh serum sample (Fig. 1, panel A) is characterized by some signals due to the presence of fibrinopeptides generated during the coagulation process: mainly fibrinopeptide A (fpA) and its degradation products (Table 1).

Indeed, during the fibrinogen-fibrin conversion mediated by the serine protease thrombin, four small peptides, two fibrinopeptides A (fpA) and two fibrinopeptides B (fpB), are cleaved from the N-terminal end of the Aα and Bβ chains of fibrinogen. Removal of these peptides leads to the exposure of cryptic polymerization sites on the fibrinogen molecule resulting in the rapid formation of a fibrin clot.

Table 1
Fibrinopeptide A (fpA) fragments ordered by increasing weight. For each fragment, its molecular weight for the mono-isotopic form is reported along with the associated sequence of aminoacids.

Peptide	$[M + H]^+$	Sequence
fpA(8–16)	905.47	FLAEGGGVR
fpA(7–16)	1020.51	DFLAEGGGVR
fpA(6–16)	1077.52	GDFLAEGGGVR
fpA(5–16)	1206.57	EGDFLAEGGGVR
fpA(4–16)	1263.59	GEGDFLAEGGGVR
fpA(3–16)	1350.62	SGEGDFLAEGGGVR
fpA(2–16)	1465.65	DSGEGDFLAEGGGVR
fpA – H_2O	1518.68	ADS(-H_2O)GEGDFLAEGGGVR
fpA	1536.69	ADSGEGDFLAEGGGVR
fpA + P	1616.65	ADSpGEGDFLAEGGGVR

It is evident that the most represented form of fpA in the MALDI-ToF spectrum of a fresh serum is the fpAY peptide (*m/z* 1465.65 $[M + H]^+$) which, compared with the full-length form, is lacking of an alanine residue. The degradation process of the full-length fpA peptide, leading to the rapid formation of fpAY, has been clearly described by Villanueva [5]. Others suggested that thrombin itself may play an important role in generating this broad spectrum proteolysis [6].

In this paper, we propose a simple method, mass spectrometry based, able to verify the integrity of serum samples by evaluating their absolute amount of fibrinopeptide A and its degradation pattern. We also introduce SeraDeg, an original software based on the proposed method and available on-line. SeraDeg is able to screen many serum spectra altogether, compute the fundamental fpA related parameters and assign a quality score to each spectrum.

2. Materials and methods

Blood samples used in this study were freshly collected from seven unrelated healthy volunteer women whose age spanned the range of 22 to 48 years. From the freshly collected samples, two sets of aliquots were generated. One set was stored at − 80 °C and analysed in triplicate 18 months later, whereas samples of the second set were subjected to analysis in triplicate, after a freeze–thaw cycle, within 2 h from collection. In the latter procedure, one vial was accidentally lost. All samples were collected and processed following a strict standardized protocol. Briefly, samples were collected in serum tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), allowed to clot at room temperature for 1 h, centrifuged at 1500*g* for 10 min, and immediately stored at − 80 °C. Studies were performed after obtaining informed consent.

As reported in our previous papers [7–9], samples were processed using Dynabeads RPC 18 beads (Invitrogen Dynal) with C18 alkyl-modified surface, which are intended for peptide (< 5000 Da) sample concentration. To set up the solid phase extraction procedure, a fixed amount of beads was tested against different amounts of a mixture of three synthetic peptides having different molecular weight and hydrophobicity to exclude both the saturation of the beads and bias due to the physicochemical properties of each peptide (data not shown). One of these peptides (MW = 1419.76) was then used as an internal standard from the elution to the MS analysis stages. Some modifications were made to the bead manufacturer's protocol. Briefly, 40 μL of beads suspension was washed one time with water and three times with 100 μL of 200 mM NaCl and 0.1% TFA. The beads were resuspended in 20 μL of water, mixed with 50 μL of serum, and left at room temperature for 5 min. After incubation, the tube was placed in the manual magnetic particle concentrator (Invitrogen Dynal) and the supernatant was discarded. The bead–peptide complex was washed three times with 300 μL of 0.1% TFA in water, and the bound peptides were eluted by incubation, at room temperature for 2 min, with 12 μL of a 1:1 acetonitrile/water solution, to which 3.5 pmol/L of the internal standard peptide was added. The standard peptide, used to normalize the data, was directly spiked in the eluting solution to avoid any interference during the binding reaction between analytes and the magnetic beads.

For AP–MALDI/ToF analysis, several parameters (matrix composition, sample/matrix ratio, spotted volume, and laser power) were tested to find the best experimental conditions. All of the spectra were acquired in a mass range from 800 to 2500 *m/z*. A quality control (QC) standard, represented by pooled human serum, was used to monitor instrument performance. QC serum was prepared with the same standardized protocol used to collect experimental samples, and an aliquot was run with every experimental sample set. Moreover, before each set of samples, the efficiency of the AP–MALDI source was verified qualitatively and quantitatively measuring both the ionization of a mix of synthetic peptides and the area of the internal standard peptide,