Chapter 1

Detection of Bacterial Protein Toxins by Solid Phase Magnetic Immunocapture and Mass Spectrometry

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Abstract

Bacterial protein toxins are involved in a number of infectious and foodborne diseases and are considered as potential biological warfare agents as well. Their sensitive multiplex detection in complex environmental, food, and biological samples are an important although challenging task. Solid-phase immunoaffinity capture provides an efficient way to enrich and purify a wide range of proteins from complex mixtures. We have shown that staphylococcal enterotoxins, for example, can be efficiently enriched by means of magnetic immunocapture using antibody functionalized paramagnetic beads. The method was successfully interfaced by the on-beads and off-beads detection using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry at the protein level and by the off-beads nano-electrospray ionization-MS/MS detection at the enzyme digests level, enabling thus the unambiguous identification of the toxin. The method is applicable to any bacterial toxin to which an antibody is available.

Key words: Bacterial protein toxins, Staphylococcal enterotoxins, Immunomagnetic separation, Immunoaffinity, Magnetic beads, Mass spectrometry, MALDI, ESI

1. Introduction

Mass spectrometry (MS) has been successfully applied in the detection, identification, quantification, and structural characterization of biological toxins (1, 2), including bacterial protein toxins, such as botulinum (3–7), shiga-like (8), tetanus (9), anthrax (10) toxins, and staphylococcal enterotoxins (SEs) (2, 11). Both matrix-assisted laser desorption/ionization (MALDI) (12) and electrospray ionization (ESI) (9) techniques have been widely exploited for the characterization of protein toxins. Their direct detection in complex biological samples like body fluids and food

Otto Holst (ed.), *Microbial Toxins: Methods and Protocols*, Methods in Molecular Biology, vol. 739,
DOI 10.1007/978-1-61779-102-4_1,

is hampered by the generally low concentration of protein toxin and by the suppression effect of the matrix. Sensitivity and specificity of these techniques can be enhanced by coupling the MS-based detection (1) to the measurement of specific enzymatic activity of the toxin by the detection/quantification of its product peptides (7), (2) to immunoaffinity enrichment/purification step (11), or by the combined application of the two (6, 10). Activity-based MS was proven to be extremely sensitive for the detection of those bacterial protein toxins which have highly specific protease activity like botulinum and anthrax toxins. Immunoaffinity enrichment, on the other hand, can be used independently from the biological activity in an unbiased way. Today, highly specific antibodies are available for a major part of bacterial protein toxins (13), which make the immunoaffinity enrichment-based MS method feasible. One of the most convenient ways to perform affinity capture is the application of antibody-coated magnetic particles as affinity probe. The major advantage of magnetic beads is that after the enrichment process they can easily be separated from complex matrices and are amenable to downstream MS-based analysis. In this chapter, we describe a procedure for the preparation of SEs immunomagnetic affinity probes and their use in MS-based detection. The method is generally applicable to other bacterial protein toxins to which antibodies are available.

2. Materials

2.1. Preparation and Use of Antibody-Coated Magnetic Particles

1. Lyophylized SEs from Toxin Technology, Inc., Sarasota, FL, USA are dissolved at 1 mg/mL in water and stored in aliquots at –20°C. SEs toxins are particularly toxic (see Note 1).
2. Polyclonal, affinity purified anti-staphylococcal enterotoxin IgGs, ≥95% IgG, from Toxin Technology, Inc. Sarasota, FL, USA, are dissolved at 1 mg/mL in water and stored in aliquots at –80°C.
3. Magnetic particles: Dynabeads® M-280, tosylactivated superparamagnetic polystyrene beads coated with a polyurethane layer are used from Dynal, Norway. Physical characteristics: diameter: 2.8 µm ± 0.2 µm, surface area: 4–8 m^2/g, active chemical functionality: 50–70 µmol/g, density: 1.3 g/cm^3, concentration: 2×10^9 beads/mL (approximately 30 mg/mL). Store at 4°C.
4. Supernatant removal and washing steps are performed using a magnetic particle concentrator from Dynal, Norway.
5. Buffer A: 0.1 M Na-phosphate buffer, pH 7.4 (2.62 g $NaH_2PO_4 \cdot H_2O$ and 14.42 g $Na_2HPO_4 \cdot 2H_2O$ dissolved in water and volume adjusted to 1,000 mL). Store at 4°C.

6. Buffer B: phosphate-buffered saline (PBS), pH 7.4, with 0.1% (w/v) bovine serum albumin (BSA) (0.88 g NaCl and 0.8 g BSA, dissolved in 0.01 M Na-phosphate pH 7.4 and volume adjusted to 100 mL). Store at 4°C.
7. Buffer C: 0.2 M Tris–HCl pH 8.5 with 0.1% (w/v) BSA (2.42 g Tris–HCl dissolved in distilled water, pH adjusted with 1 M HCl to 8.5, and volume adjusted to 100 mL). Store at 4°C.
8. Buffer D: 100 mM glycine, pH 2.5, adjusted with 1 M HCl. Store at 4°C.
9. Buffer E: same as Buffer B with 0.01% (w/v) sodium azide. Store at 4°C.

2.2. MALDI-TOF MS

1. MALDI-TOF instrument: Voyager DE-Pro (Applied Biosystems, Framingham, MA) or alternative mass spectrometer with delayed extraction and with a 337 nm nitrogen laser (see Note 2). Spectra are acquired in positive, linear acceleration mode, in the m/z 1,000–35,000 mass range. Settings: 24 kV of accelerating voltage, 76% of grid voltage and 180 ns of delay time. Averages of 10×50 laser shots are summed for one spectrum.
2. 1,2-Dimethoxy-4-hydroxycinnamic matrix (sinapinic acid, Sigma–Aldrich) is dissolved in 50% acetonitrile, 0.1% (v/v) trifluoroacetic acid solution in 10 mg/mL concentration. The matrix solution is prepared freshly. Matrix solution is light sensitive. Store at 20–22°C.

2.3. In-solution Enzymatic Digestion

1. Vivaspin 500 ultrafiltration devices with 3 kDa MWCO from Vivascience, Stonehouse, U.K. (see Note 3).
2. NH_4HCO_3. Stock solution is prepared at 100 mM concentration (see Note 4).
3. Tris[2-carboxyethyl]-phosphine-HC(TCEP)(Sigma–Aldrich). TCEP stock solution is prepared at 100 mM concentration in 100 mM NH_4HCO_3 (see Note 5). Prepare freshly.
4. Iodacetamide (IAA) (Sigma-Ultra, Sigma–Aldrich). IAA stock solution is prepared at 100 mM concentration in 100 mM NH_4HCO_3 (see Note 6). Prepare freshly before use, wrap the container with aluminum foil, and keep the solution in dark.
5. A trypsin stock solution is prepared at 100 ng/μL concentration in 5% acetic acid. Aliquots are stored at –20°C. Working solution is prepared freshly at 6 ng/μL concentration in 50 mM NH_4HCO_3. An appropriate volume of trypsin solution (2 μL) is added to the sample in order to achieve a 1:100 to 1:20 trypsin to protein ratio. Each working solution should be prepared freshly and kept on ice before use.
6. Protein LoBind Eppendorf tubes.
7. Water bath or heating block at 37°C and 100°C.

2.4. Nano-ESI-MS

1. ESI-MS instrument, QSTAR Elite (Applied Biosystems, Foster City, CA/Toronto, Canada) equipped with NanoSpray II ion source. Spectra are acquired in the positive ion mode using information-dependent analysis. Briefly, survey scans are performed in the range *m/z* 300–1,500 and the two most abundant multiply charged ions are automatically selected for MS/MS experiments. MS/MS scans are performed using nitrogen as collision gas in the range *m/z* 70–1,500 using dynamic collision energy setup. Ion source settings: 1,800 V capillary voltage, 200°C source heater, 60 V cone voltage.
2. Nano-HPLC, Ultimate 3000 Nano LC system (Dionex, Sunnyvale, CA, USA) equipped with two independent gradient pumps and an active nano flow-splitter. The instrument is connected to the ESI-MS instrument online and is set up for automated preconcentration and sample cleanup procedure using a two-position valve. The sample (10 µL) is preconcentrated and desalted by flushing solvent A through the trap column at 30 µL flow rate for 5 min. After cleanup, the two-position valve is switched to place the trap column in series with the separation column. Settings: 300 nL flow rate. Gradient: 5–50% B in 30 min 50–98% B in 6 s (see Note 7).
3. Nanoflow sprayer tip, home-made pulled silica capillary. Characteristics: 170 µm outer diameter, 100 µm inner diameter, tip 30 µm inner diameter (see Note 8).
4. Trap column, PepMap, C18, 300 µm inner diameter, 5 mm length, 300 Å pore size, and 5 µm particle size (LCPackings, Sunnyvale, CA, USA) or similar (see Note 9).
5. Separation column, PepMap, C18, 75 µm inner diameter 15 cm length, 300 Å pore size, and 3 µm particle size (LCPackings, Sunnyvale, CA, USA) or similar (see Note 10).
6. Solvent A: 2% acetonitrile in 0.1% formic acid and 0.025% trifluoroacetic acid. Solvents of highest available grade and water MilliQ or better should be used. Solvents are filtered and degassed.
7. Solvent B: 98% acetonitrile in 0.1% formic acid and 0.025% trifluoroacetic acid.

3. Methods

The method consists of four steps: (1) functionalization of the magnetic particles, (2) immunoaffinity capture of the toxin, (3) washing steps which may include elution of the toxin and the subsequent enzymatic digestion, and (4) MS detection (Fig. 1).

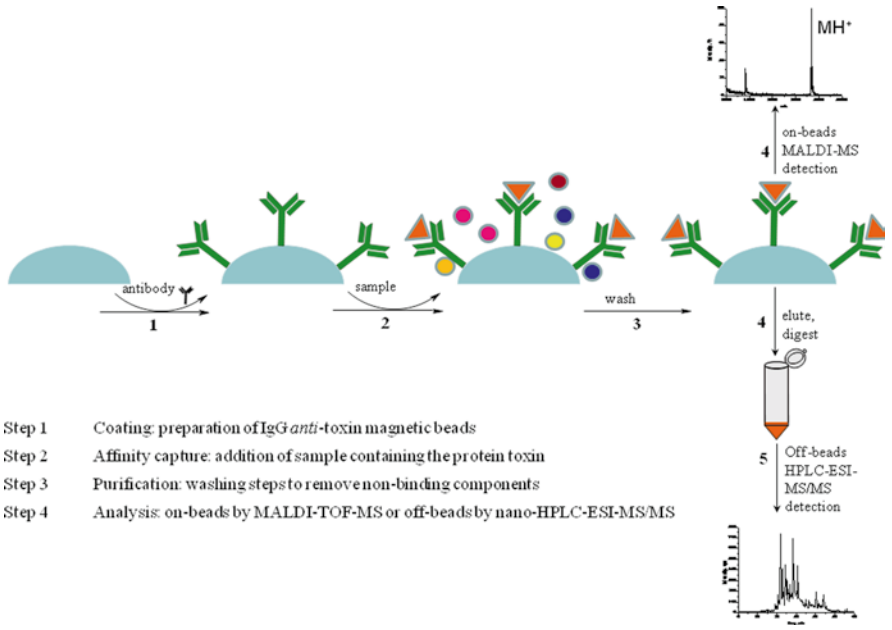

Fig. 1. Basic steps and schematics of solid phase magnetic immunocapture mass spectrometry. Symbols indicate the following: magnetic particles – *half circle*, antibody – *Y symbol*, antigen – *triangle* and impurities – *circles*.

Preparation of the affinity probe is performed by coating of monodisperse, hydrophobic magnetic particles with the antibody. It is important that the antibody is highly specific for the toxin antigen aimed to be analyzed. Antibody specificity should be evaluated by Western blot, ELISA, or MS-based pull-down assay using highly purified protein toxin antigen as standard. For the solid phase magnetic immunocapture assay, affinity-purified monoclonal antibodies are usually preferred to polyclonal ones. Antibodies can be covalently immobilized via reaction of their primary amino groups with the tosyl groups present on the magnetic beads surface. Loading capacity of the affinity probe has to be evaluated by ELISA using purified toxin standard.

In the immunoaffinity capture step, the sample is incubated and the bacterial protein toxin is selectively captured by the antibody-coated magnetic particles. After incubation, the particles are extensively washed in order to remove nonbinding molecules. The purified protein toxin bound to the particles is then either directly analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (on-beads detection) or eluted with appropriate solvent, and subsequently off-beads detected. The sensitivity of MALDI-TOF-MS for intact proteins is in the low picomol–femtomol range, depending on molecular mass and primary structure of the intact protein.

Optimization of instrument parameters, data processing, and sample preparation procedures have to be performed for each analyte. Purified toxin standard should be used to determine detection limit (Fig. 2a). In the on-beads analysis, a suspension of the beads containing the affinity-captured protein toxin is placed

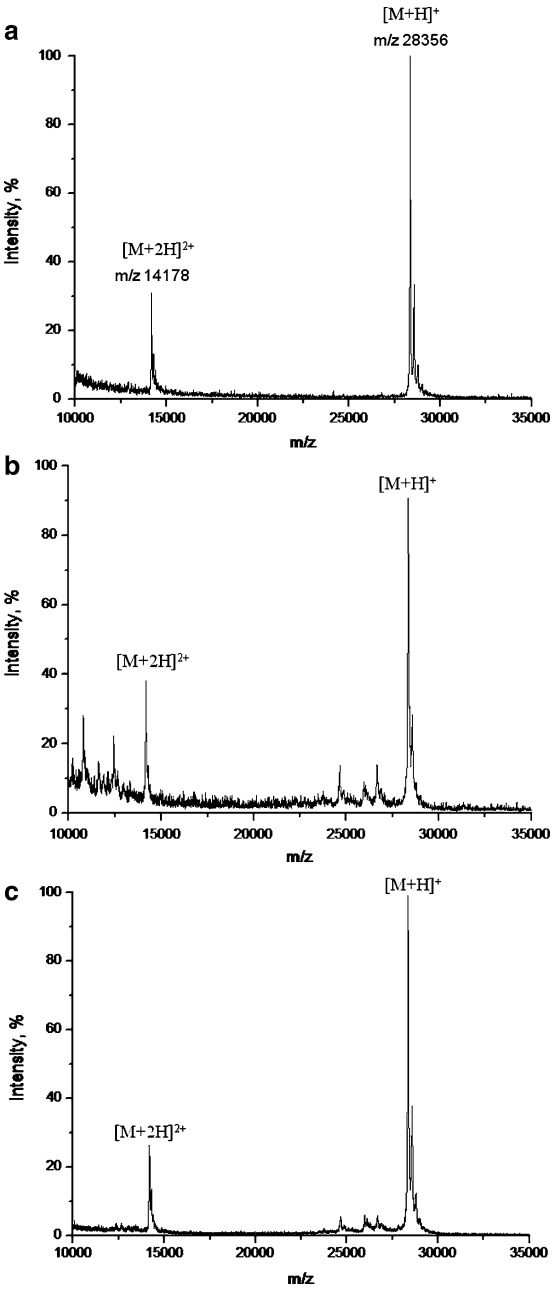

Fig. 2. MALDI-TOF mass spectrum of (**a**) SEB standard (85 fmol) and MALDI-TOF mass spectra of affinity-captured SEB in (**b**) on-beads and (**c**) off-beads detection modes.

directly onto the MALDI target plate, and the molecular mass of the protein toxin is measured in the linear mode (Fig. 2b). Magnetic beads were found to be highly compatible with MALDI under the experimental conditions typically applied for proteins. The acidic pH of the matrix (sinapinic acid) and the laser-induced desorption/ionization assist the dissociation of the antibody–antigen complex, and thus only the dissociated protein toxin can be detected in the mass spectrum. It can be easily and quickly performed by using up only small part (usually 1%) of the affinity probe. Alternatively, the protein toxin can be quickly eluted from the affinity probe by glycin at acidic pH. The presence of glycin (100 mM) in the toxin containing eluent was found not to influence negatively the subsequent MS-based analysis (Fig. 2c). The advantage of this elution step is that the affinity probe can be reused. In addition, being compatible not only with MALDI, but also with ESI, the elution step can increase the versatility of the assay. The eluted protein after pH adjustment can be proteolytically digested and analyzed by proteomics approaches which can give an additional dimension to the reliable identification and/or quantification of the toxin.

3.1. Preparation of Antibody-Coated Magnetic Particles and Immunomagnetic Isolation of Enterotoxins

1. 100 µL from the resuspended magnetic particles is washed twice with 400 µL Buffer A (see Note 11).
2. 60 µg antitoxin IgG (see Note 12) is diluted to 100 µL with Buffer A, added to the Dynabeads suspension and incubated under rotary shaking at 37°C for 24 h (see Note 13).
3. The supernatant is removed and particles are washed two times with 400 µL Buffer B at 4°C for 5 min.
4. The supernatant is removed, 400 µL Buffer C is added, and the suspension is incubated at 37°C for 4 h.
5. The supernatant is removed and the particles are washed two times with 400 µL Buffer B at 4°C for 5 min (see Note 14).
6. Particles are resuspended in the toxin-containing solution (sample) and incubated at 37°C for 1 h (see Note 15).
7. The supernatant is removed and the particles are washed five times with 400 µL Buffer B.
8. The supernatant is removed.

3.2. Online and Off-Line Detection of Bacterial Toxins Using MALDI-TOF MS

3.2.1. Online Detection

1. 1 µL suspension is loaded onto the MALDI target plate.
2. 1 µL matrix solution is added and the mixture is dried on air.
3. MALDI-TOF-MS analysis is performed. Bacterial protein toxin is identified based on the measured molecular mass.

3.2.2. Off-Line Detection

1. Particles are resuspended in 50 µL Buffer D.
2. The suspension is mixed for 1 min and the supernatant is removed (see Note 16).
3. 1 µL supernatant is mixed with 1 µL matrix solution, placed onto the MALDI target and dried on air.
4. MALDI-TOF-MS analysis is performed. Bacterial protein toxin is identified based on the measured molecular mass.

3.3. In-solution Trypsin Digestion and Off-Beads Nano-ESI-MS/MS Detection of Bacterial Toxins

1. Off-beads sample (supernatant obtained at 3.2.2.2) solution is exchanged from Buffer D to 100 mM NH_4HCO_3 using Vivaspin 500. The pH of the solution is checked after buffer exchange. In case of pH > 7.5, buffer exchange should be repeated.
2. The sample volume is adjusted to 40 µL with 100 mM NH_4HCO_3.
3. Add 5 µL TCEP stock solution and mix. Incubate the sample at 95°C for 10 min.
4. Cool down the sample to room temperature (20–22°C), add 5 µL IAA stock solution and shake in the dark for 20 min.
5. Add 2 µL working solution of trypsin, mix, and spin down.
6. Incubate for 4 h to overnight (18 h) at 37°C.
7. Acidify the sample to 2.5% final formic acid concentration using concentrated formic acid. Spin down the sample.
8. Nano-HPLC-ESI-MS/MS analysis is performed. Bacterial protein toxin is identified based on MS/MS spectra of tryptic peptide fragments.

4. Notes

1. SEs are produced by many isolates of *Staphylococcus aureus* (14). There are nine SEs (SEA–H) which have been characterized so far at the protein level, amongst which SEB is the most studied. SEB is known for causing significant nausea with vomiting, intestinal cramping, and diarrhea after hours of exposition. The toxic and lethal doses of SEB depend on the animal species and also on the route of exposure. Between 100 and 200 ng of ingested SEB can cause symptoms of staphylococcal intoxication. In humans, for aerosol exposures to SEB, currently the estimated 50% effective dose and 50% lethal dose are 0.4 and 20 ng/kg, respectively, for aerosol exposure (15).
2. Alternative similar benchtop MALDI-TOF mass spectrometers which can be used either in linear and reflectron modes are: microflex LRF (Bruker Daltonics) and M@ldi L/R (Waters, USA).

3. Vivaspin microcolumn is used according to manufacturer's instruction.
4. Prepare freshly and keep at 4°C.
5. TCEP is a valid alternative protein reduction agent to the commonly used dithiothreitol or 2-mercaptoethanol. TCEP is nonvolatile (odorless), more stable, and more effective than traditional reduction agents.
6. IAA is light-sensitive.
7. For HPLC, filtered (0.22 μm) and degassed solvents should be used. Solvents should be replaced weakly.
8. Similar nano-flow electrospray tips suitable for online ESI analysis are commercially available from New Objective, Woburn, MA USA.
9. Alternative commercial reverse phase C18 trap columns are (1) Symmetry 300 C18 NanoEase (Waters, USA) and (2) ZORBAX 300SB C18, 0.3×5 mm, 5-μm particles (Agilent Technologies, USA). Similar columns can be custom or homemade as well.
10. Alternative commercial reverse phase C18 nano-HPLC column is ZORBAX 300 SB C18, 75 μm×150 mm, 3.5-μm particles (Agilent Technologies, USA). Similar columns can be custom or home made as well.
11. High viscosity of the sample hinders the sedimentation of the magnetic particles.
12. Antitoxin IgGs are preferably affinity-purified and free of other proteins. These bind to the surface and reduce capacity of the magnetic particles.
13. All washing and incubation steps are performed under strong agitation to avoid sedimentation of the particles.
14. Antibody coated Dynabeads can be stored in Buffer D at 4°C for maximum 60 days. Before reuse, particles are washed five times with 400 μL Buffer B at 4°C for 5 min.
15. Volume of the sample is preferably below 1 mL.
16. Prolonged presence of antitoxin-coated particles in Buffer D causes loss of binding capacity.

References

1. Seto Y, Kanamori-Kataoka M (2005) Mass spectrometric strategy for the determination of natural and synthetic organic toxins. J Health Sci 51:519–525
2. Brun V, Dupuis A, Adrait A, Marcellin M, Thomas D, Court M, Vandenesch F, Garin J (2007) Isotope-labeled protein standards: toward absolute quantitative proteomics. Mol Cell Proteomics 6:2139–2149
3. Kalb SR, Moura H, Boyer AE, McWilliams LG, Pirkle JL, Barr JR (2006) The use of Endopep-MS for the detection of botulinum toxins A, B, E, and F in serum and stool samples. Anal Biochem 351:84–92

4. Hines HB, Lebeda F, Hale M, Brueggemann EE (2005) Characterization of botulinum progenitor toxins by mass spectrometry. Appl Environ Microbiol 71:4478–4486
5. Kalb SR, Moura H, Boyer AE, McWilliams LG, Pirkle JL, Barr JR (2006) The use of Endopep-MS for the detection of botulinum toxins A, B, E, and F in serum and stool samples. Anal Biochem 351:84–92
6. Kalb SR, Lou J, Garcia-Rodriguez C, Geren IN, Smith TJ, Moura H et al (2009) Extraction and inhibition of enzymatic activity of botulinum neurotoxins/A1, /A2, and /A3 by a panel of monoclonal anti-BoNT/A antibodies. PLoS One 4:5355
7. Boyer AE, Moura H, Woolfitt AR, Kalb SR, McWilliams LG, Pavlopoulos A et al (2005) From the mouse to the mass spectrometer: detection and differentiation of the endoproteinase activities of botulinum neurotoxins A-G by mass spectrometry. Anal Chem 77:3916–3924
8. Williams JP, Green BN, Smith DC, Jennings KR, Moore KAH, Slade SE et al (2005) Noncovalent Shiga-like toxin assemblies: characterization by means of mass spectrometry and tandem mass spectrometry. Biochemistry 44:8282–8290
9. van Baar BLM, Hulst AG, Roberts B, Wils ERJ (2002) Characterization of tetanus toxin, neat and in culture supernatant, by electrospray mass spectrometry. Anal Biochem 301:278–289
10. Boyer AE, Quinn CP, Woolfitt AR, Pirkle JL, McWilliams LG, Stamey KL et al (2007) Detection and quantification of anthrax lethal factor in serum by mass spectrometry. Anal Chem 79:8463–8470
11. Schlosser G, Kacer P, Kuzma M, Szilagyi Z, Sorrentino A, Manzo C et al (2007) Coupling immunomagnetic separation on magnetic beads with matrix-assisted laser desorption ionization-time of flight mass spectrometry for detection of staphylococcal enterotoxin B. Appl Environ Microbiol 73:6945–6952
12. Bernardo K, Fleer S, Pakulat N, Krut O, Hünger F, Krönke M (2002) Identification of *Staphylococcus aureus* exotoxins by combined sodium dodecyl sulfate gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Proteomics 2:740–746
13. Pauly D, Kirchner S, Stoermann B, Schreiber T, Kaulfuss S, Schade R et al (2009) Simultaneous quantification of five bacterial and plant toxins from complex matrices using a multiplexed fluorescent magnetic suspension assay. Analyst 134:2028–2039
14. Pocsfalvi G, Cacace G, Cuccurullo M, Serluca G, Sorrentino A, Schlosser G et al (2008) Proteomic analysis of exoproteins expressed by enterotoxigenic *Staphylococcus aureus* strains. Proteomics 8:2462–2476
15. Papageorgiou AC, Tranter HS, Acharya KR (1998) Crystal structure of microbial superantigen staphylococcal enterotoxin B at 1.5 A resolution: implications for superantigen recognition by MHC class II molecules and T-cell receptors. J Mol Biol 277:61–79