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Chapter 5

Comparison of sample preparation protocols for the analysis of the *Bacillus cereus* spore and cell proteome

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Abstract

The spore is a resistant form of life in *Bacillus cereus*. Special structure and composition make spore proteome analysis challenging. In this study, a recent “single tube” approach, single-pot solid-phase-enhanced sample preparation (SP3), that uses sodium dodecyl sulfate (SDS) for protein extraction was applied for extracting proteins from *B. cereus* spores and vegetative cells. The performance of SP3 protein and peptide clean-up methods were compared with the well-established “one-pot” method regarding reproducibility, proteome coverage and specific compartment protein coverage. All three methods performed similarly on vegetative cell samples. SP3 protein clean-up was slightly better in identification of proteins and peptides from spore samples with the maximum number of 1852 for proteins and 10851 for peptides. The SP3 protein clean-up showed the best reproducibility for spore sample. In conclusion, the SP3 protein clean-up method was successfully applied on *B. cereus* vegetative cell proteome and had an enhanced performance on the analysis of the spore proteome.

5.1. Introduction

As a strategy for gram-positive spore-former species Bacilli and Clostridia to survive harsh conditions, bacterial spores are formed through a developmental process called sporulation [1]. Life is kept in a highly dehydrated spore core, which is protected by multiple layers, from inside to outside, inner membrane, germ wall, peptidoglycan cortex, outer membrane, proteinaceous coat and exosporium which is a specific layer produced by certain bacteria [2]. In a favorable environment, germinant receptors located in the inner membrane of dormant spores recognize small nutrients, which initiates germination followed by outgrowth [3]. Proteomics analysis can help in gaining a deeper understanding of the spore's molecular mechanisms involved both in its formation during sporulation as well as in its transformation back to a vegetative cell upon germination [4].

Bottom-up mass spectrometry-based proteomics is widely used on complex mixtures, using peptides as a proxy to identify and quantify proteins [5]. A typical sample preparation workflow for bottom-up proteomics includes cell or tissue disruption, protein extraction, enzymatic digestion, peptide purification and recovery prior to analysis. Every step, in the preparation of a protein sample for bottom-up proteomics can significantly alter the representation of proteins in the final peptide mixture [6-10]. Both the protein extraction as well as subsequent conditions for enzymatic digestion and peptide clean-up can in principle affect the proteomic analysis of a sample. To date many protocols to prepare cellular, tissue or other sample types for proteomics analysis have been published. These protocols differ in their use of protein solubilizing agents (e.g. tri-fluoro-ethanol, urea, guanidine, dodecyl sulfate, deoxycholate) and how these often interfering agents are removed prior to mass spectrometric analysis. Well established modern sample preparation methods are, filter assisted sample preparation [11, 12] (FASP), in stage-tip digestion [13] (iST) and single-pot solid-phase-enhanced sample preparation [7, 14-20] (SP3). In addition to these, more classic protocols involving gel-based sample preparation [21-29], protein precipitation [30-34] and different in solution preparation protocols using various (mass spectrometry compatible) surfactants or chaotropic agents [35-40] are also still in common use.

In contrast to cellular proteomics, studying the spore proteome is challenging due to the inherently resistant spore structure and the large portion of insoluble proteins present in a spore [41]. The "one-pot" method has been especially developed for high-throughput spore proteomics [42], offering a detergent-free method using the chaotrope urea for protein

extraction. This “one-pot” method has served as a standard method in studying proteome wide sporulation and germination dynamics in *Bacillus subtilis* [43, 44], and has also been applied to *Clostridium difficile* cells and spores [45]. However, due to the aforementioned large portion of insoluble proteins present in spores and their multiple membrane structures, use of strong detergents could be advantageous in spore sample preparation by enhancing protein extraction and solubilization. To circumvent the inherent incompatibility of most detergents with mass spectrometric analysis we chose to use SP3 to remove detergents prior to mass spectrometric analysis, while maintaining the “single vessel” and high-throughput characteristics of the “one-pot” method. We adapted the original protocols for the cleanup of proteins and peptides [17] from detergents to optimize their use in bacterial cell and spore proteomics workflow (Figure 5.1). In this study, we compare the performance of SP3 protein- and peptide-cleanup on spore and cell samples of *B. cereus* (a spore forming human pathogen) and compare it to the gold standard “one pot” method regarding quantitative reproducibility, proteome coverage and spore specific protein identification.

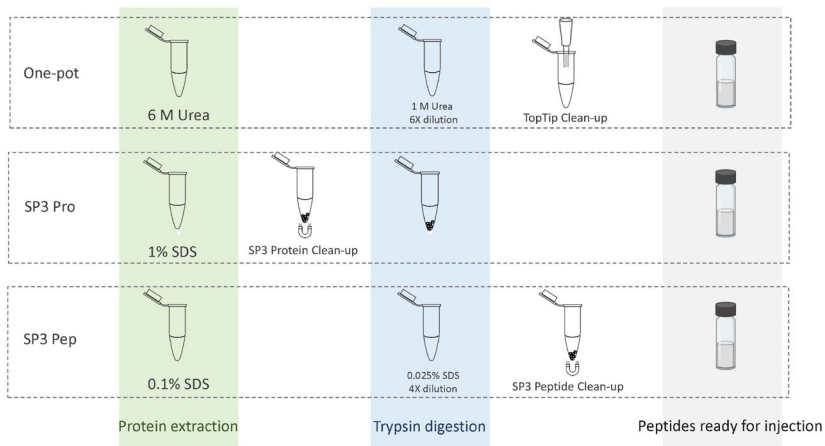


Figure 5.1. Workflow of “one-pot”, SP3 protein clean-up (SP3 Pro) and SP3 peptide clean-up (SP3 pep) methods. The starting point for all three methods is protein extraction. Proteins from equal amounts of spores or vegetative cells were extracted by bead-beating with different extraction buffers. Extracted proteins were digested into peptides by trypsin after dilution in “one-pot” and SP3 Pep workflows. The peptides were either purified by top-tip clean-up in the “one-pot” method or by magnetic beads in SP3 Pep method. Extracted proteins in SP3 Pro were first purified using magnetic beads and then digested with trypsin.

5.2. Results

5.2.1. Comparison of “one-pot” and SP3 for the analysis of *B. cereus* cells and

spores

Following the adaptation of SP3 protein and peptide-cleanup to fit into our established cell and spore lysis protocol using a bead mill (Figure 5.1) we compared the performance of these sample preparation methods to our established “one-pot” protocol on *B. cereus* cells and spores. Using the “one-pot” method, we identified 1132 proteins (10206 peptides) from the cellular samples, compared to 1039 (8967 peptides) for SP3 protein cleanup and 1015 proteins (7408 peptides) for SP3 peptide cleanup (Figure 5.2A and 2B) for the three biological replicates tested. For the spore samples we identified 1500 proteins (8000 peptides) with the one-pot” method, 1449 proteins (10851 peptides) with the SP3 protein- and 1021 proteins (5990 peptides) with the peptide-cleanup method (Figure 5.2C and 2D). Apart from the number of proteins identified the reproducibility of protein and peptide quantitation was also assayed. The coefficient of variation (CV) of cellular

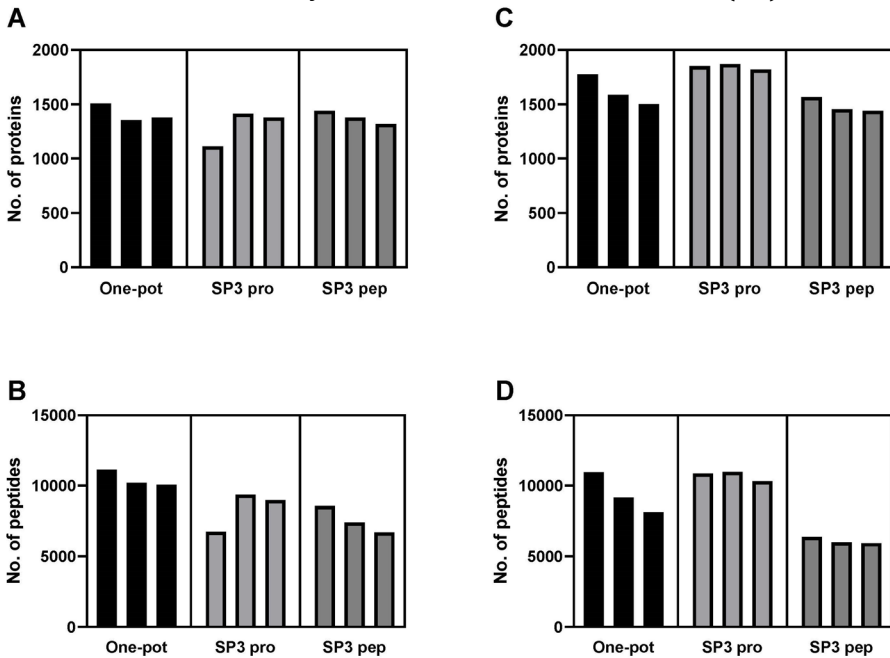


Figure 5.2. Comparison of “One-pot”, SP3 protein clean-up and SP3 peptide clean-up regarding numbers of identified proteins and peptides from cells (panel A, B) and spores (panel C, D).

proteins, was similar with median CVs of 0.85% (“one-pot”), 0.93% (SP3 protein-cleanup) and 0.89% (SP3 peptide-cleanup). Reproducibility of cellular peptides had median CVs of 17.20% (“one-pot”), 16.38% (SP3 protein-cleanup) and 19.10% (SP3 peptide-cleanup). For spore samples

median CVs of 1.02% (“one-pot”), 0.57% (SP3 protein-cleanup) and 1.05% (SP3 peptide-cleanup) for protein quantitation were found. While peptides from spore samples had median CVs of 19.34% (“one-pot”), 10.08% (SP3 protein-cleanup) and 20.34% (SP3 peptide-cleanup). This already shows that especially the SP3 protein-cleanup shows very similar performance with regard to numbers of proteins and peptides identified and their reproducibility of quantitation from cellular or spore samples.

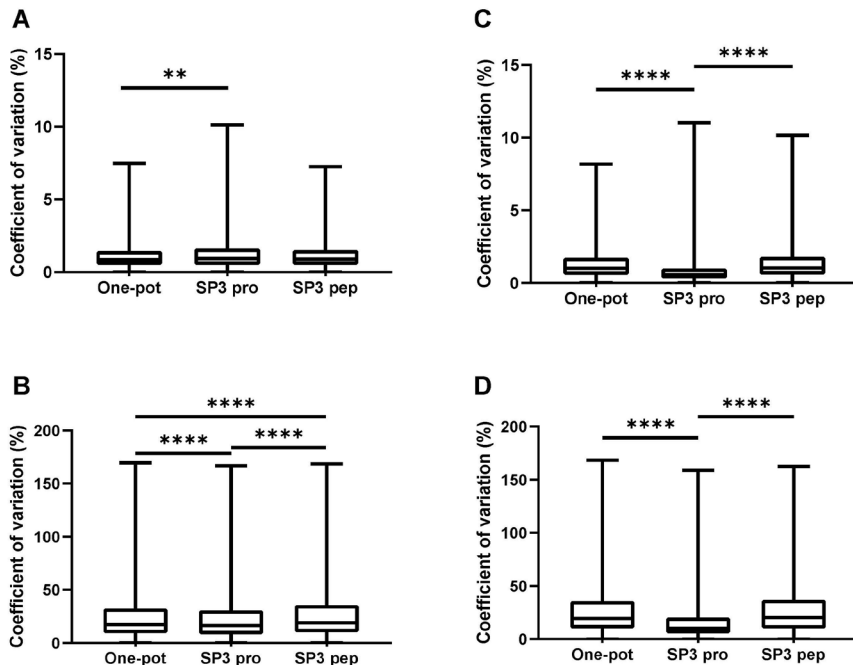


Figure 5.3. Coefficient of Variation (CV) for proteins and peptides quantified. (A, B) The identified cellular proteins and peptides. (C, D) The identified spore proteins and peptides. **, $p < 0.01$, ****, $p < 0.0001$.

The majority of the proteins were identified by all three protocols. The overlap among three protocols of the total of 1267 cellular proteins was 68% (857) and of the 1612 spore proteins was 55% (880) as shown in Figure 5.4A and 5.4C. The one-pot method identified slightly more proteins in cellular samples while SP3 protein cleanup identified most proteins in spore samples. To determine whether certain classes of proteins were predominantly identified by any one protocol, gene ontology cellular component (GOCC) analysis was carried out on proteins identified by the three methods. Among the cellular samples, the “one-pot” method identified slightly more cytosolic proteins. The SP3 protein cleanup identified less proteins belonging to the bacterial-type flagellum hook and basal body

categories. For spore samples, SP3 protein clean-up identified more spore cytosolic and membrane proteins. Overall, there were no large differences in the protein class identified by the different methods (Figure 5.4B and 4D).

We further investigated whether there were differences in the abundance of identified proteins by the three methods. Proteins identified by all three protocols were classified using K-means clustering and each cluster was functionally enriched by GOCC analysis (Figure 5.5). For cellular samples, cluster K1 consisted of a larger portion of the integral component of membrane and plasma membrane proteins corresponding with higher intensities in SP3 protein and peptide cleanup. Cluster K2 and K3 contain a larger part of cytosol proteins which correspond with higher intensities in the “one-pot” method. For spore samples, cluster K2 contained a higher fraction of membrane associated proteins, which also correlated to higher intensities in SP3 protein and peptide cleanup.

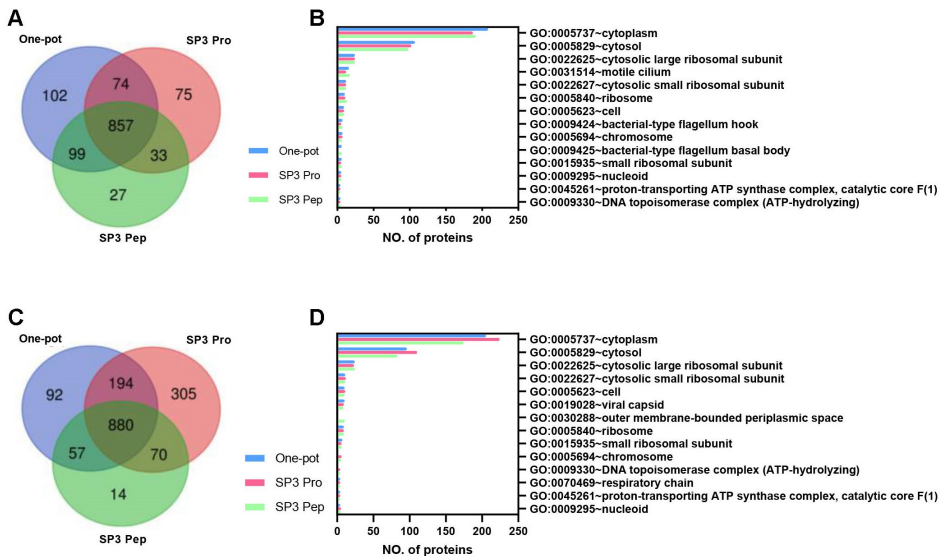


Figure 5.4. Venn diagram of identified proteins by “One-pot”, SP3 Pro and SP3 Pep (A, C) and bar plot of the number of proteins with indicated “gene ontology cellular component (GOCC)” category annotations (B, D). (A, B) The cell samples. (C, D) The spore samples.

5.2.2. Protein coverage comparison of spore specific proteins.

Germination is an essential step in the progression of spore revival. Many efforts are put towards studying germination and proteins that are involved in germination [46], especially the germinant receptors [47-50] that trigger spore germination. Germinant receptors are crucial but very difficult to extract, because of their low abundance and the highly compressed spore

inner membrane where they localize [51]. We estimated the number of germination associated proteins identified by the three methods to estimate their performance for extraction of proteins localized at inner membrane. The germinant receptors and germination proteins identified in this study are listed in Table 5.1. In total, 173, 163 and 111 peptides of germination

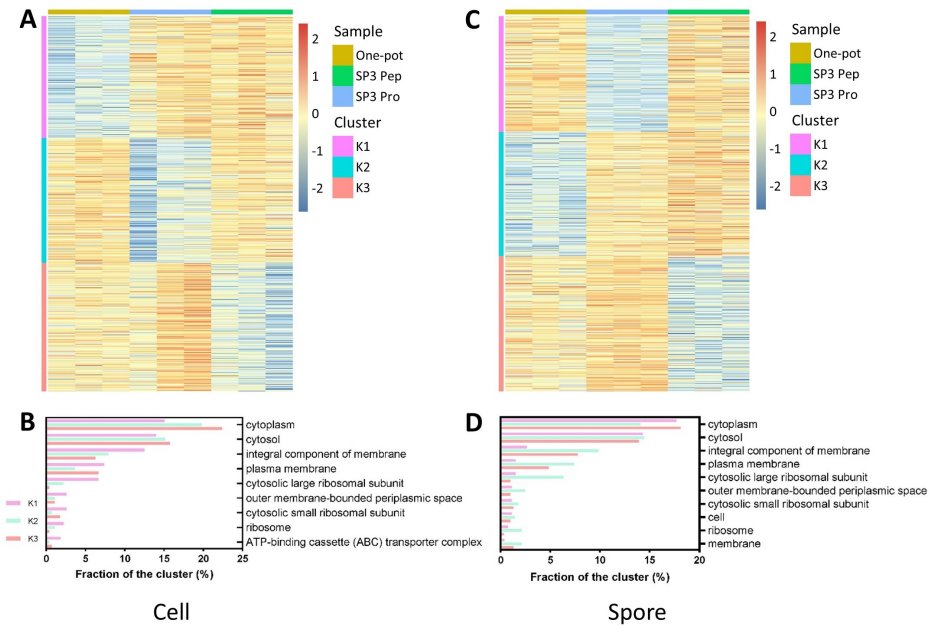


Figure 5.5. Heat map of identified proteins by three protocols and functional “cellular component” enrichment of each cluster. The heat map of proteins identified by three methods from cell samples (A) and spore samples (C). Proteins were clustered into 3 clusters (K1, K2 and K3) by K-means clustering. The bar graph of GOCC functional analysis of K1-K3 clusters from cell samples (B) and spore samples (D).

associated proteins were identified by “one-pot”, SP3 protein- and SP3 peptide-cleanup, respectively. Although more peptides were identified using the “one-pot” method, three more germination associated proteins were identified by SP3 protein-cleanup. Only the SP3 protein cleanup method identified two germinant receptor A subunits (GerSA and GerRA) and a C subunit (GerLC), but failed to identify GerPC which were identified by the other two protocols. However, all three protocols cannot identify all germinant receptors of *B. cereus*. The coat layer is specific for spores with varieties of protection. Proteins are cross linked in the coat layer [52], making it harder for protein extraction. A special layer for *B. cereus* spores is assembled outside of the coat layer, called the exosporium. The proteins belonging to the spore coat and exosporium layers identified by the three

methods are listed in Table 5.2. The list of spore proteins from the coat compartment was established based on a previous study [41]. For coat and exosporium protein identification, the three protocols performed similarly. 55 proteins were identified by “one-pot” and SP3 peptide clean-up, and 56 proteins were identified by SP3 protein clean-up. But “one-pot” identified 639 unique peptides in total, much higher than the number of peptides identified by SP3 protein cleanup (455) and SP3 peptide cleanup (387).

Table 5.1. Germination associated proteins identified in this study. *, ** and *** represent the number of replicates of proteins were identified. NA, not identified.

Gene	Uniprot Code	Total no. of unique peptides			Identified in this study		
		"One-pot"	SP3 pro	SP3 pep	"One-pot"	SP3 pro	SP3 pep
<i>gerIA</i>	Q816T6	8	9	4	***	***	***
<i>gerIC</i>	Q816T4	3	7	3	***	***	***
<i>gerSA</i>	Q81AJ2	0	3	0	NA	***	NA
<i>gerSC</i>	Q81AJ0	1	1	0	*	*	NA
<i>gerRA</i>	Q81HM0	0	3	0	NA	***	NA
<i>gerRC</i>	Q81HM1	0	1	1	NA	**	**
<i>gerLC</i>	Q81HS6	0	2	0	NA	***	NA
<i>gerM</i>	Q817P2	2	4	3	*	***	***
<i>gerPC</i>	Q81GP7	6	0	2	***	NA	***
<i>gerQ</i>	Q814N4	7	4	6	***	***	***
<i>yaaH</i>	Q81AG3	34	27	22	***	***	***
<i>ypeB</i>	Q813I5	22	23	16	***	***	***
<i>spoVAC</i>	Q815K0	2	4	0	**	***	NA
<i>spoVAD</i>	Q819B9	6	5	2	***	***	***
<i>spoVAD</i>	Q815J9	12	9	4	***	***	***
<i>spoVAE</i>	Q819C0	6	5	2	NA	NA	NA
<i>spoVAE</i>	Q819C1	0	2	3	NA	**	***
<i>spoVAF</i>	Q819C2	9	10	4	***	***	***
<i>gerD</i>	Q81J09	11	11	14	***	***	***
<i>htrC</i>	Q814H6	21	17	13	***	***	***
<i>sleB</i>	P0A3V0	13	9	6	***	***	***
<i>cwIj</i>	Q814N5	10	7	6	***	***	***
<i>cwIj</i>	Q81D46	2	0	1	***	NA	*

Table 5.2. Spore coat and exosporium proteins identified in this study. *, ** and *** represent the number of replicates of proteins were identified. NA, not identified.

Gene	Total no. of unique peptides	Identified in this study
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	Uniprot Code	"One-pot"	SP3 pro	SP3 pep	"One-pot"	SP3 pro	SP3 pep
<i>yabG</i>	Q81JA4	11	10	8	***	***	***
<i>yabP</i>	Q81J89	9	4	6	***	***	***
<i>yusW</i>	Q81IY1	15	12	8	***	***	***
<i>cotB</i>	Q81IJ7	18	9	7	***	***	***
<i>cotB</i>	Q81IJ6	12	5	6	***	***	***
<i>cotJC</i>	Q81HI7	7	5	4	***	***	***
<i>cotJB</i>	Q81HI6	4	4	4	***	***	***
<i>cotJA</i>	Q81HI5	5	3	2	***	***	***
<i>cotY</i>	Q81GH8	5	2	1	***	***	***
<i>BC_1245</i>	Q81GF8	5	5	3	***	***	***
<i>cotN</i>	Q81GC8	23	17	16	***	***	***
<i>spoIVA</i>	Q81FR0	30	21	16	***	***	***
<i>yppG</i>	Q81FM1	4	1	1	***	***	**
<i>cotD</i>	Q81FM0	1	0	1	*	NA	*
<i>BC_2030</i>	Q81EF0	6	4	2	***	***	***
<i>ytfJ</i>	Q81E93	15	7	5	***	***	***
<i>BC_2677</i>	Q81CR9	14	11	7	***	***	***
<i>cotX</i>	Q81CA3	9	6	7	***	***	***
<i>cotX</i>	Q81CA1	6	6	6	***	***	***
<i>yxeE</i>	Q81AM8	4	4	4	***	***	***
<i>cotE</i>	Q81A24	15	8	8	***	***	***
<i>dacF</i>	Q819B1	23	16	16	***	***	***
<i>yhcN</i>	Q817V9	26	16	17	***	***	***
<i>safA</i>	Q812R2	19	22	24	***	***	***
<i>ytfJ</i>	Q817B7	17	6	8	***	***	***
<i>BC_5056</i>	Q815S6	18	14	19	***	***	***
<i>BC_3621</i>	Q81AF1	1	1	0	*	**	NA
<i>BC_2873</i>	Q81CA2	3	1	0	***	***	NA
<i>BC_2031</i>	Q81EE9	0	4	2	NA	***	**
<i>cota</i>	Q819D8	8	3	2	***	***	***
<i>BC_4639</i>	Q817B8	13	10	10	***	***	***
<i>yqfX</i>	Q81G20	3	5	3	***	***	***
<i>yqfX</i>	Q81E89	3	2	2	***	***	***
<i>yisY</i>	Q816P9	7	9	3	***	***	***
<i>exsY</i>	Q81GI1	9	4	4	***	***	***

<i>exsK</i>	Q81D85	8	7	4	***	***	***
<i>bclC</i>	Q812Y5	9	6	4	***	***	***
<i>bxpB</i>	Q813V0	5	3	4	***	***	***
<i>BC_3547</i>	Q81AL6	7	4	2	***	***	***
<i>exsFB</i>	Q813L4	2	1	1	***	*	**
<i>bclB</i>	Q81DI4	0	0	0	NA	NA	NA
<i>BC_2639</i>	Q81CV2	8	3	1	***	***	*
<i>BC_3345</i>	Q81B46	0	1	1	NA	***	**
<i>BC_2569</i>	Q81D14	9	3	2	***	***	***
<i>bxpA</i>	Q81E43	6	3	2	***	***	***
<i>inA</i>	Q81GC3	34	20	21	***	***	***
<i>BC_2267</i>	Q81DT6	5	3	2	***	***	***
<i>BC_2266</i>	Q81DT7	5	4	3	***	***	***
<i>calY</i>	Q81GC6	19	15	13	***	***	***
<i>yaaH</i>	Q81AG3	35	27	22	***	***	***
<i>gerQ</i>	Q814N4	7	4	6	***	***	***
<i>alr1</i>	Q81IT5	30	22	16	***	***	***
<i>iunH</i>	Q81C90	14	10	8	***	***	***
<i>BC_2207</i>	Q81DY9	16	9	10	***	***	***
<i>gpr</i>	Q818E2	10	11	5	***	***	***
<i>BC_1591</i>	Q81FJ1	6	7	3	***	***	***
<i>iunH</i>	Q81AL1	14	12	9	***	***	***
<i>ypeB</i>	Q813I5	22	23	16	***	***	***

5.3. Discussion

To study the diverse and complex proteome, a wealth of protocols have been developed to collect and process samples from different biological systems for enhancement in proteome coverage depth, along with optimizations on proteolytic digestion and peptide recovery. However, the efficiency and reproducibility are always under consideration for a robust and sensitive proteomic workflow. The popularity of a single vessel sample preparation approach is more efficient, by both avoiding potential sample losses and simplifying the workflow at the same time. In the present study, both “one-pot” and SP3 methods are single-tube and labor-saving approaches for high-throughput proteome analysis, together with trypsin-based “in-solution” digestion, these approaches are more readily automatable and efficient compared with “in-gel” digestion [17]. In the cellular context the “one-pot” method identified the most proteins and peptides although the difference

with the SP3 protein-cleanup was not large. The SP3 protein-cleanup did identify significantly more spore proteins and peptides compared to the other two methods. The performance of SP3 peptide cleanup was lower in numbers of proteins and peptides identified in both sample contexts. All three methods showed a good reproducibility, based on the CV of protein and peptide abundances within the expected range of label free quantitation. Based on GOCC analysis, although the three methods did not show a large preference to protein classes identified, the “one-pot” method had an overall lower intensity level of integral membrane proteins. The lower intensity levels of the membrane proteins in the “one-pot” method are presumably due to the lack of detergents in this approach. In the spore context, SP3 protein-cleanup identified slightly more germinant receptors, and similar numbers of coat and exosporium proteins, although peptide coverage was higher for these proteins using the “one-pot” method.

One of the main distinctions of “one-pot” and SP3 is the option of protein solubilizing agents that can be used, urea versus SDS, which may be a reason for more identified spore proteins by the SP3 protein clean-up methods. The urea-trypsin in-solution digestion is presumably less efficient in denaturation of membrane proteins [53]. Another drawback of urea is that it can cause carbamylation of proteins which prevents full tryptic digestion [54]. Using the hydrophilic magnetic beads to aggregate proteins and peptides can alleviate interprotein aggregation [53] improving digestion efficiency [15]. However, this there is a potential for loss of information on unbound proteins and peptides, which could be the reason for the lower number of identified peptides from coat and exosporium layers by both SP3 protein and peptide cleanup. The overall lower performance of SP3 peptide cleanup could be due to the lower initial concentration of SDS and subsequent dilution to a compatible concentration for the proteolytic digestion. The lower concentration of SDS may not be sufficient for protein extraction and solubilization resulting in the lower performance of SP3 peptide-cleanup. In this study none of the three methods identified all germinant receptors, so other extraction approaches could be investigated to see if these would add to a more complete coverage of membrane bound protein structures. Possible avenues would be using in solution digest approaches using mass spectrometry compatible surfactants such as Proteasemax, RapiGest SF, PPS Silent Surfactant and Invitrosol. Another recent development that could be of interest in improving spore proteomics is the newly developed SPEED method. Here samples are solubilized using high amounts of trifluoro-acetic acid to lyse cells and break down non-proteinaceous constituents in a single vessel high throughput approach. This

has already been applied to *B. cereus* cells and could be potentially very useful in spore proteomics [35].

In conclusion, sample preparation is a vital step that affects quality of quantitative proteomic data, especially for the spore proteome. In this study, we evaluated the performances of “one-pot” and SP3 protein clean-up and SP3 peptide clean-up on the analysis of spore and vegetative cell proteome of *B. cereus*. The SP3 protein clean-up method was as effective as the “one-pot” method for the vegetative cell proteome and has a slightly better performance for the spore proteome. The SP3 protein clean-up protocol can be applied on cells and spores allowing an increased throughput and depth in the study of life cycle of spore forming organisms.

5.4. Materials and Methods

5.4.1. Strain and culture

For spore samples, strain *B. cereus* ATCC 14579 was cultured and sporulated as described previously [41]. A single colony was inoculated into tryptic soy broth (TSB) liquid medium and cultured at 30 °C overnight. Cells were harvested by centrifugation and washed before transfer to chemically defined growth and sporulation (CDGS) medium. After four-days of sporulation, spores were harvested and washed for at least 4 times with cold Milli-Q water. Cell samples were prepared by harvesting exponential phase cells from TSB liquid medium. Three biological replicates of spores and vegetative cells samples were prepared for testing each protocol.

5.4.2. Sample preparation

The details of the three sample preparation protocols are shown in Figure 5.1. Sample lysis was adapted from the “one-pot” method described previously [42] to the three protocols using different lysis buffers. Spores of $OD_{600} = 2$ or cells of $OD_{600} = 0.8$ were suspended in 200 μ l of the protocol specific lysis buffer. The lysis buffer contained 10 mM TCEP (Tris (2-carboxyethyl) phosphine hydrochloride), 30 mM CAA (2-Chloroacetamide) and 100 mM ammonium bicarbonate (AmBiC) with 6 M urea for “one-pot”, 1% SDS for the SP3 protein- and 0.1% SDS for the SP3 peptide-cleanup method. All samples were disrupted with 0.9 g 0.1 mm zirconium-silica beads (BioSpec Products, Bartlesville, OK, USA) using a Precellys 24 (Bertin Technologies, Aix en Provence, France). The sample disruption program was set to run seven rounds with 20 s for each round and a 60-seconds interval. Samples were cooled down on ice every three rounds to avoid overheating. The concentrations of extracted proteins from spore and cell lysates were determined using the Pierce™ BCA assay kit (Thermo Fisher Scientific,

Waltham, MA, USA).

Since the chaotrope (urea) and detergents (SDS) in the lysis buffer affect tryptic digestion efficiency, they were either diluted in “one-pot” and SP3 peptide-cleanup or removed in SP3 protein-cleanup. The beads for SP3 were a mixture of two kinds of magnetic carboxylate modified beads (Sera-Mag™ Magnetic carboxylate modified particles (Hydrophilic), CAT NO. 24152105050250, and Sera-Mag™ Magnetic carboxylate modified particles (Hydrophobic), CAT NO. 44152105050250) with a ratio of 1:1 before use and prepared as described previously [17]. For SP3 protein-cleanup samples, around 20 µg protein lysates were mixed with 2 µl prepared bead mix in PCR tubes. Acetonitrile (ACN) was immediately added to a final concentration of 50% (v/v). After incubation for 18 min at room temperature, beads were immobilized by settling on a magnetic rack for 2 min. Subsequently we removed the supernatant and washed with 200 µl 70% (v/v) ethanol twice and 180 µl ACN once. Finally, air dried beads were reconstituted and incubated with 10 µl 100 mM AmBiC with addition of trypsin at an enzyme to protein ratio of 1:50 at 37 °C overnight. Peptides were recovered by acidifying supernatant with formic acid (FA) to a final concentration of 1%, which was ready for downstream injection onto the mass spectrometer.

Sample lysates from “one-pot” and SP3 peptide-cleanup methods were diluted with 100 mM AmBiC for 6 times and 4 times, respectively. Trypsin was supplemented with an enzyme to protein ratio of 1:100. The mixtures were incubated at 37 °C overnight. For one-pot method, digestion was quenched with addition of trifluoroacetic acid (TFA). Peptides were purified and recovered using C18 reversed- phase TT2 Top-Tips (Glygen). For SP3 peptide clean-up, 40 µl of tryptic digest peptide mixture was mixed with 2 µl beads mix. After addition of ACN to a final concentration of 95%, samples were incubated for 18 min and 2 min on a magnetic rack at room temperature. The supernatant was discarded. Samples were washed with 180 µl ACN twice and peptides were eluted with 0.1% formic acid in water.

Samples (200ng) were injected by a Ultimate 3000 RSLCnano UHPLC system (Thermo Scientific, Germeringen, Germany) onto a 75 µm × 250 mm analytical column (C18, 1.6 µm particle size, Aurora, Ionopticks, Australia) kept at 50 °C at 400 nl/min for 15 min in 3% solvent B before being separated by a multi-step gradient (Solvent A: 0.1% formic acid in water, Solvent B: 0.1% formic acid in acetonitrile) to 5% B at 16 min, 17% B at 38 min, 25% B at 43 min, 34% B at 46 min, 99% B at 47 min held until 54 min returning to initial conditions at 55 min equilibrating until 80 min.

Eluting peptides were sprayed by the emitter coupled to the column into a

captive spray source (Bruker, Bremen Germany) with a capillary voltage of 1.5 kV, a source gas flow of 3 L/min of pure nitrogen and a dry temperature setting of 180 °C, attached to a timsTOF pro (Bruker, Bremen Germany) trapped ion mobility separation, quadrupole, time of flight mass spectrometer. The timsTOF was operated in PASEF mode of acquisition. The TOF scan range was 100-1700 m/z and a tims range of 0.6-1.6 V.s/cm². In PASEF mode a filter was applied to the m/z and ion mobility plane to select features most likely representing peptide precursors, the quad isolation width was 2 Th at 700 m/z and 3 Th at 800 m/z, and the collision energy was ramped from 20-59 eV over the tims scan range to generate fragmentation spectra. A total of 10 PASEF MS/MS scans scheduled with a total cycle time of 1.16 seconds, scheduling target intensity 2e4 and intensity threshold of 2.5e3 and a charge state range of 0-5 were used. Active exclusion was on (release after 0.4 min), reconsidering precursors if ratio current/previous intensity > 4.

5.4.3. Data Processing

Raw data was processed in Maxquant (version: 1.6.14.0) [55] and searched against *Bacillus cereus* ATCC 14579 database (09-2019) downloaded from Uniprot [56]. Trypsin/P was selected as the protease for protein digestion and a maximum of 2 missed cleavages were allowed. The carbamidomethylation (C) was set as a fixed modification and oxidation as a variable modification. The “Match between runs” was selected, and in the “Label-free quantification” module, the LFQ option was selected.

The “proteinGroup.txt” was loaded into Perseus (version: 1.6.15.0) [57] and LFQ intensity was used for data analysis. The analysis was carried out separately on spore and cell samples. Proteins identified at least two replicates were analyzed for number of identified proteins, coefficient of variation and functional enrichment.

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