Identification and Monitoring of Host Cell Proteins by Mass Spectrometry Combined with High Performance Immunochemistry Testing

Katrin Bomans¹, Antje Lang², Veronika Roedl², Lisa Adolf¹, Kyrillos Kyriosoglou¹, Katharina Diepold¹, Gabriele Eberl², Michael Melhøj¹, Ulrike Strauss², Christian Schmalz², Rudolf Vogel³, Dietmar Reusch¹, Harald Wegele¹, Michael Wiedmann¹, Patrick Bulau*¹

¹ Pharma Development, Roche Diagnostics GmbH, Penzberg, Germany, ² Pharma Biotech, Roche Diagnostics GmbH, Penzberg, Germany, ³ Professional Diagnostics, Roche Diagnostics GmbH, Penzberg, Germany

Abstract

Biotherapeutics are often produced in non-human host cells like Escherichia coli, yeast, and various mammalian cell lines. A major focus of any therapeutic protein purification process is to reduce host cell proteins to an acceptable low level. In this study, various E. coli host cell proteins were identified at different purifications steps by HPLC fractionation, SDS-PAGE analysis, and tryptic peptide mapping combined with online liquid chromatography mass spectrometry (LC-MS). However, no host cell proteins could be verified by direct LC-MS analysis of final drug substance material. In contrast, the application of affinity enrichment chromatography prior to comprehensive LC-MS was adequate to identify several low abundant host cell proteins at the final drug substance level. Bacterial alkaline phosphatase (BAP) was identified as being the most abundant host cell protein at several purification steps. Thus, we firstly established two different assays for enzymatic and immunological BAP monitoring using the cobas® technology. By using this strategy we were able to demonstrate an almost complete removal of BAP enzymatic activity by the established therapeutic protein purification process. In summary, the impact of fermentation, purification, and formulation conditions on host cell protein removal and biological activity can be conducted by monitoring process-specific host cell proteins in a GMP-compatible and high-throughput (> 1000 samples/day) manner.

Introduction

Host cell proteins (HCPs) carry potential clinical safety risks for patients treated with biologics. On the one hand, HCP might cause an immune response (due to their “non-self” nature), adjuvant activity, and theoretically also function in the human body [1–3]. Furthermore, HCPs with protease activity have the potential to impact product stability [4]. Consequently, regulatory guidelines mandate the setting of HCP specifications [5].

Thus, one key aspect of any biologics manufacturing is to reduce HCP to levels considered acceptable in the final drug [6]. The HCP composition is impacted by the proteome complexity of the utilized host expression system [7–9], the manner in which the therapeutic protein is expressed [10–13], and the purification process itself [10,12]. Moreover, all methods for analytical HCP characterization face challenges due to the dynamic range of HCP abundance at proteome and final drug level.

Several analytical techniques have been used for the detection, identification, and quantification of HCPs [1,3,14,15]. To perform bio-process and release analytics, immunoassays like protein gel blots and multicomponent generic or process-specific enzyme-linked immunosorbent assays (ELISA) are most commonly used to detect and monitor HCPs [16–18]. The ELISA technique is typically applied for HCP analysis, mostly due to the good precision of the method and also that it provides quantitative results for setting control limits and specifications. However, generic ELISAs do not offer complete coverage for all process-specific HCPs and process-specific
ELISAs might not be qualified to evaluate the HCP content after process changes [3,16–18]. Two-dimensional gel electrophoresis combined with fluorescent staining is also applied for the detection and quantification of HCPs [19,20]. The technique is semi-quantitative, has a limited dynamic range, and needs mass spectrometry for HCP identification.

Approaches involving liquid chromatography coupled to mass spectrometry (LC-MS) provide alternative solutions for product characterization within the biopharmaceutical industry [21–25]. Advances in two dimensional LC-MS (2D–LC-MS) have enabled the analysis of low-abundance analytes in complex protein mixtures [26–28]. Recently, the identification and quantification of HCPs in biotherapeutics by 2D–LC-MS was demonstrated [29,30].

In the present study, an approach employing affinity chromatography to capture HCPs, highly sensitive LC-MSMs, and high throughput immunoassay testing for the enrichment, identification and quantification of HCPs in biotherapeutics was developed. This test system allowed us to identify and monitor Bacterial Alkaline Phosphatase in a biopharmaceutical purification process.

Results

Increased levels of HCPs were detected in the manufacturing process for a recombinant protein derived from E. coli by ELISA and RP-HPLC analysis. At final drug substance level, several batches with HCP levels minimal greater than the release specification of 30 ppm were observed by a process-specific ELISA system. RP-HPLC analysis with UV detection is routinely applied to monitor product variants at the final drug substance level and at various purifications steps (Figure 1). At the final drug substance level no significant differences in product purity where observed for batches with elevated HCP levels (Figure 2A). The first chromatographic purification step of the recombinant protein is accomplished by metal chelate chromatography (purification step 1). At this stage several product variants (marked by asterisks) can be observed in the obtained elution pool (Figure 2B).

In general, no significant differences in product variants where observed for batches with elevated HCP levels at purification step 1 level. However, a slight but distinct increase in peak intensity of the product variant with a retention time of 25 min could be observed for batches with elevated HCP levels (Figure 2B; marked by an arrow). The existence of elevated product variants or HCP levels was further suggested by SDS-PAGE analysis of the respective HPLC fractions (retention time window: 24-26 min), in which an increased content of a protein species with a molecular weight of around 23 kDa was identified as the protein, 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase, and N-acetylmuramoyl-L-alanine amidase AmiD were only detected at final drug substance level (Figure 2C). However, the Protein tolB, DNA protection during starvation C). Since BAP was identified as being the most abundant HCP suggesting a relative enrichment of the protein through the purification process. Thus, from a qualitative point of view, the results achieved do not suggest significant alterations of the HCP profile related to the affinity enrichment step.

Next, we employed the described approach to identify HCPs at the final drug substance level. A total of 12 bacterial proteins (with an identification score ≥ 20), not detected without affinity enrichment, were identified at final bulk level (Table 3). Eight proteins were already verified at purification step 1 elution pool level (including BAP and Alkyl hydroperoxide reductase subunit C). However, the Protein tolB, DNA protection during starvation protein, 2-amino-4-hydroxy-6-hydroxymethylidihydropteridine pyrophosphokinase, and N-acetylmuramoyl-L-alanine amidase Aminotransferase were only detected at final drug substance level suggesting a relative enrichment of the protein through the purification process.

In summary, the application of affinity chromatography combined with comprehensive LC-MS was adequate to identify low abundant HCPs at the final drug substance level. Since BAP was identified as being the most abundant HCP at purification step 1 elution pool level and is still traceable at final drug substance level we developed two different assays
for enzymatic and immunological BAP monitoring on a cobas INTEGRA® 400 plus and a cobas e 411 system, respectively. The method validation results for both cobas® systems demonstrated acceptable analytical performance and are in accordance with the manufacturer’s accuracy and imprecision criteria (Table 4). The cobas® analysis of all total purification step elution pools and the final drug substance material does demonstrate an almost complete BAP removal by the applied purification process (Table 5). At final drug substance level the detected immunological BAP activity of 0.1 U/mg was close to the quantification limit (0.12 U/mL) and the observed enzymatic BAP activity was below the detection limit of 0.2 mU/mL (see Table 4). As initially described, several batches with increased HCP levels were observed at final drug substance level by a process-specific ELISA system. To verify if BAP does contribute to the elevated total HCP levels the enzymatic and immunological BAP activity of various batches was analyzed at purification step 3 elution pool level. The results are
summarized in Table 6 and demonstrate that no causal relationship between elevated total HCP levels and enzymatic and immunological BAP activity is verifiable.

**Discussion**

For the identification and quantification of HCPs in biotherapeutics, a test system employing HCP affinity enrichment, highly sensitive LC-MS, and high throughput immunoassay testing was developed. Direct LC-MS analysis was capable of identifying 76 HCPs at purification step 1 level but did not verify the presence of HCPs at the final drug substance level. In contrast, the application of affinity chromatography for HCP enrichment combined with LC-MS was adequate to identify 85 HCPs at purification step 1 level. In addition, the results achieved do not suggest, from a qualitative point of view, significant alterations of the HCP profile related to the affinity enrichment step. The described approach resulted in the confirmation of 12 low abundant HCPs in the final drug substance. All identified bacterial HCPs have the potential to trigger an immune response in human, although the respond will depend on composition and amount of bacterial proteins administered [2,3]. Moreover, five low abundant bacterial proteins with possible catalytic activity were verified.

Bacterial Alkaline Phosphatase (BAP) is a widely distributed non-specific phosphomonoesterase that also catalyzes phosphoryl transfer reaction to various alcohols [31,32]. *E. coli* Alkyl hydroperoxide reductase (AhpC) directly reduces organic hydroperoxides in its reduced dithiol form and might act as an antioxidant enzyme [33]. 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine pyrophosphokinase, Adenylate kinase, and N-acetylmuramoyl-L-alanine amidase AmiD were also detected at the final drug substance level. The identified bacterial enzymes are involved in folic acid biosynthesis.
Figure 3. SDS-PAGE analysis of RP-HPLC fractions. (a) Mark12™ Standard; (b) Reference material, drug substance level; (c) RP-HPLC fraction (24-26 min, Figure 2B) of purification step 1 elution pool, HCP content at drug substance level: 13 ppm; (d) RP-HPLC fraction (24-26 min, Figure 2B) of purification step 1 elution pool, HCP content at drug substance level: 35 ppm.

doi: 10.1371/journal.pone.0081639.g003
levels of some drug substance batches. The results revealed that no causal relationship between elevated total HCP levels and cell wall biogenesis/degradation [34–37]. The described catalytic activities do not suggest a negative impact on product stability. However, bacterial enzymes could theoretically also have enzymatic effects in humans, although, to the author’s knowledge, reports to this risk have not been published [3]. BAP, AhpC, and Adenylate kinase were detected at purification step 1 and final drug substance level whereas 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase and N-acetylMuramoyl-L-alanine amidase AmiD were only detected at the final drug substance level. Thus, the data indicate a less effective removal or relative enrichment of both enzymes through the purification process.

Subsequently, we focused on developing test systems for high throughput monitoring of specific bacterial HCPs. Since BAP was identified as being the most abundant HCP at purification step 1 level, we firstly established two different assays for enzymatic and immunological BAP monitoring using the cobas® technology. The obtained results demonstrate an almost complete removal of BAP enzymatic and immunological activity by the applied purification process. However, since BAP enzymatic activity was verified until purification step 3 level BAP activity at the final drug substance level is most likely, although to a much lower extent. These results are in agreement with the data obtained by comprehensive LC-MS analysis, in which BAP at final bulk level was only found after enrichment by affinity chromatography. In addition, we assessed if BAP does contribute to the elevated total HCP levels of some drug substance batches. The results revealed that no causal relationship between elevated total HCP levels and enzymatic and immunological BAP activity is traceable. Currently, we are developing novel immunological and enzymatic cobas® test systems to assess AhpC, 2-amino-4-hydroxy-6-hydroxymethylidihydropteridine pyrophosphokinase, Adenylate kinase, and N-acetylMuramoyl-L-alanine amidase AmiD removal by the applied purification process and perhaps more importantly, their catalytic activity at the final drug substance level. Recently, the identification and quantification of HCPs in biotherapeutics by 2D–LC-MS was demonstrated [29,30]. The described approaches allow the simultaneous quantification of various low abundance HCPs. In the present study, the application of affinity chromatography combined with comprehensive LC-MS analysis and cobas® technology was adequate to identify low abundant HCPs at final drug substance level and to subsequently monitor the enzymatic and immunological activity of BAP at various purifications steps. Accordingly, the impact of fermentation, purification, and formulation conditions on HCP removal and biological activity can be conducted by monitoring process-specific HCPs in a GMP-compatible and high-throughput (> 1000 samples/day) manner.

### Materials and Methods

**Reversed Phase Chromatography (RP-HPLC)**

Reversed phase chromatography was performed on a Dionex Summit® HPLC system (Thermo Fisher Scientific, Bremen, Germany) with UV detection at 210 nm. The separation was carried out on a YMC-Pack ODS-AQ analytical column (3 x 150 mm, S-3μ, 200 Å, carbon content: 11.0-11.5 %, YMC, Tokyo, Japan) between 20°C and 25°C. A step gradient using 0.1% TFA, 30 % acetonitrile as solvent A and 0.1 % TFA, 80 % acetonitrile as solvent B at 0.4 mL/min was applied. For the chromatographic analysis 6 μg of total protein was injected. Fractions were collected manually.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

One-dimensional SDS-PAGE was performed with Novex® 18% Tris-Glycine gels in a XCell SureLock® Mini-Cell (Life Technologies Corporation, Darmstadt, Germany). Samples were reduced with NuPAGE® Sample Reducing Agent (catalogue number: NP0004) and gel electrophoresis was

---

**Table 1. Overview of identified HCPs at different purification (P.) steps.**

| ACC   | Identified E. coli protein | MW [kDa] | P. step 1 | P. step 2 | P. step 3 | Drug substance |
|-------|---------------------------|----------|-----------|-----------|-----------|---------------|
| P0634 | Alkaline phosphatase      | 49.4     | 458       | 92        | 210       | 88            | n.d. n.d.     |
| P0A87B| Chaperone protein dnaK    | 69.1     | 250       | 80        | 151       | 61            | n.d. n.d.     |
| P23843 | Periplasmic oligopeptide-binding protein | 60.9 | 214 | 78 | 107 | 68 | n.d. n.d. n.d. n.d. |
| P0A681 | Elongation factor Ts     | 30.4     | 163       | 76        | 87        | 60            | n.d. n.d.     |
| P0A899 | Enolase                  | 45.6     | 116       | 69        | 70        | 50            | n.d. n.d.     |
| P0A877 | Tryptophan synthase alpha chain | 28.7 | 103 | 87 | 42 | 51 | n.d. n.d. n.d. n.d. |
| P0AE08 | Alkyl hydroperoxide reductase subunit C | 20.8 | 97 | 84 | 74 | 84 | n.d. n.d. n.d. n.d. |
| P0AFM2 | Glycine betaine-binding periplasmic protein | 36.0 | 92 | 79 | <20 | 20 | n.d. n.d. n.d. n.d. |
| P0ABK5 | Cysteine synthase A      | 34.5     | 84       | 76        | 22        | 29            | n.d. n.d.     |
| P0AFM7 | Uncharacterized protein ybbN | 31.8 | 68 | 58 | 52 | 62 | n.d. n.d. n.d. n.d. |
|       | Total number of identified HCPs (score >20) |       | 76       | 16        | 1        | 0             |

Database query was conducted by analyzing LC-MS/MS CID spectra using Proteome Discoverer V1.3 and a false discovery rate FDR < 1%. The Top 10 HCPs were sorted according to the score value at purification step 1 level. ACC, accession number (http://www.uniprot.org/); SC, score; MW, theoretical molecular weight; COV, Sequence coverage; n.d., not detected.

doi: 10.1371/journal.pone.0081639.t001
carried out according to the Novex® Tris-Glycine Midi Gels instruction (quick reference card, 25-0913 Version B; 12 May 2008). 5 µg of protein was loaded per lane. Low concentrated fractions from reverse phase chromatography were evaporated to dryness in a RVC 2-25 CD plus Rotational-Vacuum-Concentrator (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The gels were stained with SimplyBlue™ SafeStain (Life Technologies Corporation) and destained in ultrapure water.

Figure 4. SDS-PAGE analysis of in-process controls. (a) Mark12™ Standard; (b) Purification step 1 elution pool; (c) Purification step 2 elution pool; (d) Purification step 3 elution pool; (e) Drug substance, HCP content: 30 ppm. doi: 10.1371/journal.pone.0081639.g004
Table 2. Comparison of identified HCPs with and without HCP affinity enrichment.

| Identified E. coli protein | Direct analysis | After HCP enrichment |
|---------------------------|----------------|----------------------|
| MW [kDa] | SC | COV [%] | SC | COV [%] |
| P00834 Alkaline phosphatase | 49.4 | 458 | 92 | 251 | 83 |
| P0A6Y6 Chaperone protein dnaK | 69.1 | 214 | 78 | 155 | 64 |
| P23843 Periplasmic oligopeptide-binding protein | 60.9 | 30.4 | 66 | 76 | 22 |
| P0A6P1 Elongation factor Ts | 56.8 | 56 | 28 | 90 | 63 |
| P0A6P9 Enolase | 45.6 | 116 | 69 | 60 | 83 |
| P0A877 Tryptophan synthase alpha chain | 28.7 | 103 | 87 | 115 | 76 |
| P0AE08 Alkyl hydroperoxide reductase subunit C | 20.7 | 97 | 84 | 71 | 84 |
| P0AFM2 Glycine betaine-binding periplasmic protein | 36.0 | 214 | 78 | 155 | 64 |
| P0ABK5 Cysteine synthase A | 34.5 | 94 | 67 | 75 | 64 |
| P77395 Uncharacterized protein ybbN | 31.8 | 68 | 28 | 73 | 63 |
| P0A6P7 Probable GTP-binding protein engB | 23.5 | 65 | 68 | 78 | 77 |
| P0A799 Phosphoglycerate kinase | 41.1 | 64 | 45 | 90 | 63 |
| P9180 Antigen 43 | 106.8 | 62 | 21 | 39 | 16 |
| P0A862 Thiol peroxidase | 17.8 | 60 | 76 | 53 | 76 |
| P9993 Carbon storage regulator | 6.9 | 56 | 33 | <20 | 33 |
| Total number of identified HCPs (score >20) | 76 | 84 |

Database query was conducted by analyzing LC-MS/MS CID spectra using Proteome Discoverer V1.3 and a false discovery rate FDR < 1%. ACC, accession number (http://www.uniprot.org/); SC, score; MW, theoretical molecular weight; COV, Sequence coverage.

doi: 10.1371/journal.pone.0081639.t002

In-gel proteolytic digestion

In-gel digestion was carried out using OMX-S® devices (OMX GmbH, Martinsried, Germany) according to the OMX-S® pro Instruction Manual. Protein bands were washed and destained with 50% acetonitrile, 50% ammonium bicarbonate solution (50 mM, pH 8.0). The digest was performed in 20 µL freshly prepared trypsin solution (0.01 µg/µl) in 50 mM ammonium bicarbonate solution (pH 8.0) at 37°C for 45 min.

HCP identification by Nano ESI-MS/MS

For the Nano ESI-MS/MS analysis of the in-gel digests, peptides were desalted and concentrated with ZipTip® C18 Pipette Tips (Millipore Corporation, Billerica, USA) and eluted in 20 µL 1% formic acid, 50% acetonitrile. Nano ESI-MS/MS analyses were performed on a QTOF Ultima mass spectrometer (Waters, Manchester, U.K.) equipped with a TriVersa NanoMate (Advion, Inc., Ithaca, USA). Spectra were recorded in the positive ion mode. Sequencing was performed by low-energy collision-induced dissociation (CID) using argon as collision gas. The collision energy was set from 20 to 45 eV. Spectra were searched against the UniProt database of Escherichia coli using Protein Prospector (UCSF, University of California, San Francisco; http://prospector.ucsf.edu/prospector/mshome.htm) and Mascot (Matrix Science Inc, Boston, USA; http://www.matrixscience.com/search_form_select.html).

Table 3. Identified HCPs at drug substance level after HCP affinity enrichment.

| ACC | Identified E. coli protein | MW [kDa] | SC | COV [%] |
|-----|---------------------------|----------|----|--------|
| P23843 | Periplasmic oligopeptide-binding protein | 60.9 | 93 | 60 |
| P0A6Y9 | Chaperone protein dnaK | 69.1 | 77 | 40 |
| P23847 | Periplasmic dipeptide transport protein | 60.3 | 77 | 46 |
| P00634 | Alkaline phosphatase | 49.4 | 48 | 43 |
| P0A85S | Protein tolB | 45.9 | 42 | 37 |
| P0ABT2 | DNA protection during starvation protein | 18.7 | 38 | 60 |
| P0A8F5 | 60 kDa chaperonin | 57.3 | 32 | 22 |
| P0AE08 | Alkyl hydroperoxide reductase subunit C | 20.7 | 26 | 47 |
| P26281 | 2-amino-4-hydroxy-6-hydroxymethyl-5,6-dihydropteridine pyrophosphokinase | 18.1 | 26 | 58 |
| P69441 | Adenylate kinase | 23.6 | 22 | 36 |
| P75820 | N-acetylmuramoyl-L-alanine amidase AmiD | 31.1 | 21 | 30 |
| P0A910 | Outer membrane protein A | 37.2 | 20 | 26 |
| Total number of identified HCPs (score >20) | 12 | |

Database query was conducted by analyzing LC-MS/MS CID spectra using Proteome Discoverer V1.3 and a false discovery rate FDR < 1%. ACC, accession number (http://www.uniprot.org/); SC, score; MW, theoretical molecular weight; COV, Sequence coverage.

doi: 10.1371/journal.pone.0081639.t003

Table 4. Analytical performance of the BAP ECLIA and BAP enzymatic activity assay.

| Instrument | cobas e 411 | cobas INTEGRA® 400 plus |
|-----------|------------|-------------------------|
| Assay | BAP ECLIA | BAP Enzymatic activity |
| Detection Limit [mU/mL] | 40 | 0.2 |
| Quantitation Limit [mU/mL] | 120 | 1.0 |
| Range [mU/mL] | 120 - 15 000 | 1.0 - 1200 |
| Accuracy: Recovery of spike [%] | 90 - 118 | 72 - 105 |
| Precision: Repeatability RSD [%] | < 10 | 1 - 12 |
| Performance | 100 samples/120 min | 100 samples/35 min |
| Dilution of samples | Done by system | Done by system |
| BAP | Bacterial Alkaline Phosphatase; ECLIA, Electrochemiluminescence Immunobord assay. |

doi: 10.1371/journal.pone.0081639.t004

Monitoring of Host Cell Proteins

November 2013 | Volume 8 | Issue 11 | e81639
Monitoring of Host Cell Proteins

Table 5. Immunological and enzymatic BAP activities at different process levels.

| Protein | HCP ELISA BAP | ECLIA BAP | Enzymatic activity |
|---------|----------------|-----------|-------------------|
|         | [ng/mL]        | [ng/mg]   | [U/mg]            |
| Purification Step 1 | 1.44 n.a. | 8390 | 970 |
| Purification Step 2 | 0.28 n.a. | 38 | 0.7 |
| Purification Step 3 | 6.44 | 264 | 9.0 | 0.4 |
| Drug substance | 1.74 | 16 | 0.1 | <DL |

BAP, Bacterial Alkaline Phosphatase; ECLIA, Electrochemiluminescence Immunoassay; n.a., not applicable; DL, Detection limit

doi: 10.1371/journal.pone.0081639.t005

Table 6. HCP content and BAP enzymatic and immunological activities of various drug substance batches.

| Drug substance | Purification step 3 | Protein | BAP ELISA | BAP ECLIA | BAP Enzymatic activity |
|----------------|---------------------|---------|-----------|-----------|------------------------|
|                | [ng/mL]             | [ng/mg] | [U/mg]    | [mU/mg]   |
| Batch 3        | 15                  | 6.61    | 25        | 0.3       |
| Batch 4        | 32                  | 5.78    | 17        | 0.3       |
| Batch 5        | 22                  | 5.70    | 19        | 0.3       |
| Batch 6        | 30                  | 6.13    | 20        | 0.2       |
| Batch 7        | 19                  | 6.23    | 18        | 0.2       |
| Batch 8        | 20                  | 6.56    | 13        | <QL       |
| Batch 9        | 19                  | 6.40    | 18        | 0.2       |
| Batch 10       | 32                  | 7.57    | 27        | 0.4       |
| Batch 11       | 36                  | 7.42    | 18        | 0.3       |
| Batch 12       | 8                   | 6.78    | 22        | 0.2       |

BAP, Bacterial Alkaline Phosphatase; ECLIA, Electrochemiluminescence Immunoassay; QL, Quantification limit

doi: 10.1371/journal.pone.0081639.t006

In-solution digestion

For the proteolytic digestion, samples were denatured in 0.4 M Tris-HCl, 8 M guanidine hydrochloride, pH 8.5 by diluting 150 µg of protein in a total volume of 300 µL. For reduction, 10 µL of 0.1 g/mL dithiothreitol was added followed by incubation at 50°C for 1 hour. After alkylation of free cysteine by adding 10 µL of 0.33 g/mL iodoacetic acid and incubation at room temperature under exclusion of light for 30 min, the buffer was exchanged to digestion buffer (0.1 M Tris-HCl, pH 8.5) by application onto an illustra™ NAP™-5 gel filtration column (GE Healthcare, Buckinghamshire, UK). The NAP™-5 eluate (500 µL) was mixed with 30 µL of a solution of 0.1 mg/mL sequencing grade trypsin (Promega, Madison, USA) in 10 mM HCl and incubated at 37°C for 18 h.

HCP identification by LC-MS/MS

LC-MS/MS analysis of in solution digests were performed on a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to an Acquity UPLC system (Waters, Manchester, UK). Peptides were separated on a BEH C18 column (1.7 µm 2.1x150 mm, Waters, Manchester, UK) using a binary gradient (solvent A: 0.1% formic acid, solvent B: 0.1% formic acid, 100% acetonitrile) from 1% to 45% B at a flow rate of 0.3 mL/min in 40 min. 5 µg of digested protein was loaded. Low concentrated digests were evaporated to 30 µL in a RVC 2 - 25 CD plus Rotational-Vacuum-Concentrator and completely injected. Data acquisition was controlled by XCalibur software (Thermo, Waltham, MA). For the top10 CID method, survey full scan MS spectra (from m/z 200 - 2000) were acquired by the Orbitrap system with a resolution of r = 30000. The ten most abundant peptide ions with charge states > +1 were sequentially isolated and fragmented with CID and a normalized collision energy of 40 V using helium as collision gas. The resulting fragment ions were detected by the ion trap. The automatic gain control (AGC) target values were set to 1*10⁶ for Full MS scans in the Orbitrap mass analyzer and 1*10⁴ for MS/MS scans in the ion trap mass analyzer.

LC-MS/MS database query

CID spectra were searched against an in-house E. coli database using Proteome Discoverer V1.3 (Thermo Fisher Scientific, Bremen, Germany). The custom protein database was compiled from 5,967 E.coli protein sequences (from actual Swiss Prot K12 database), the sequence of the therapeutic protein, and the sequence of porcine trypsin. The following SEQUEST® search parameters were applied: (1) trypsin with a maximum of one missed cleavage, (2) carboxymethylation of cysteines as fixed and (3) oxidation of methionine as variable modifications. Precursor mass tolerance was set to 10 ppm and the fragment mass tolerance was set to 0.8 Da. A False Discovery Rate (FDR) of 1% was calculated. Only proteins specified with a score value ≥ 20 and with high confidence unique peptides (FDR < 1%) were considered as positive hits. The mass spectrometry proteomics data for HCP identification at drug substance level have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository[38,39] with the dataset identifier PXD000509 and DOI 10.6019/PXD000509.

HCP enrichment by affinity chromatography

For the preparation of the affinity resin, NHS activated sepharoseTM 4 fast flow (GE Healthcare, Buckinghamshire, UK) and anti-E. coli antibody (Dako Deutschland GmbH, Hamburg, Germany) were used. The antibody solution was dialyzed against coupling buffer (200 mM potassium phosphate, pH 8.4) and adjusted to a concentration of ca. 10 mg/mL. Coupling was performed with an adapted procedure derived from technical note 71 5000 14 AD (GE Healthcare, Buckinghamshire, UK). Unspecific binding sites of the resin were blocked by incubating with drug substance solution. The prepared affinity resin was filled into a Kronlab Eco glass column (10 x 250 mm, YMC Europe GmbH, Dinslaken, Germany) and regenerated by the following steps: (1) 0.5 M NaCl, 0.05 % Tween20, pH 7.5, 4 CV (column volumes); (2) 30 mM NaCl, 4 CV; (3) 1 M propionic acid, 2 CV; (4) 50 mM potassium phosphate, 150 mM NaCl pH 7.5, 2 CV. 2.2 mg of drug substance solution containing 80 µg HCP were loaded on
the affinity resin. Column equilibration was performed with 10 mM potassium phosphate, 154 mM NaCl, 0.005 % NaN₃, 0.05 % Triton-x 100, 0.1 % BSA pH 7.0, 4 CV followed by washing step 1 with 10 mM potassium phosphate, 100 mM NaCl, 0.005 % NaN₃, 0.05 % Tween20, 0.5 mM EDTA, pH 6.5 (4 CV) and washing step 2 using 10 mM potassium phosphate, 30 mM NaCl, pH 7.5 (4 CV). HCPs were eluted with 3 CV of 1 M propionic acid. Flow rate was set to 0.15 ml/min. The eluate was collected as a single fraction in potassium phosphate (pH 7.5) and was adjusted to pH 7.5. To remove propionic acid the eluate was dialyzed against 10 mM potassium phosphate, 154 mM NaCl, pH 7.5.

Quantitative determination of the enzymatic activity of alkaline phosphatase

The colorimetric assay was carried out on a cobas INTEGRA® 400 plus system using the ALP IFCC Gen.2 cassettes (Roche Diagnostics Ltd., Basel, Switzerland) according to the manufacturer's guidelines. Briefly, the principle of the assay is the cleavage of p-nitrophenyl phosphate by alkaline phosphatase into phosphate and p-nitrophenol. The p-nitrophenol released is directly proportional to the catalytic alkaline phosphatase activity. The activity is determined by measuring the increase in absorbance at 409 nm. The assay is performed at 37°C. A minimal sample volume of 250 µL was required. Calibration was done with Bacterial Alkaline Phosphatase (Life Technologies GmbH, Darmstadt, Germany, Catalogue number 18011-015).

Bacterial Alkaline Phosphatase (BAP) ECLIA

The immunological BAP content was determined by an electrochemiluminescence immunoassay (ECLIA) on a cobas e 411 analyzer (Roche Diagnostics Ltd., Basel, Switzerland) according to the manufacturer's guidelines. Briefly, the assay is based on a sandwich principle using a mouse monoclonal anti-BAP antibody (in-house development). First, 30 µl of sample, biotinylated anti-BAP capture antibody and ruthenium-labeled anti-BAP detection antibody were incubated for 9 min forming a ternary complex. In a second step after addition of streptavidin-coated microparticles, the complex is bound to the solid phase via interaction of biotin and streptavidin during 9 min of incubation. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured to the surface of the electrode. Voltage-induced chemiluminescence is measured by a photomultiplier. The concentration of BAP in the test sample is finally calculated from a BAP (Life Technologies GmbH, Darmstadt, Germany, Catalogue number 18011-015) standard curve of known concentration.

Supporting Information

Table S1. Repeatability of HCP identification results. Database query was conducted by analyzing LC-MS/MS spectra using Proteome Discoverer V1.3 and a false discovery rate FDR < 1% of three purification step 1 elution pool samples (three independent sample preparations). The Top 15 HCPs were sorted according to the score value of Analysis 1. Accession number, http://www.uniprot.org; MW, theoretical molecular weight.

Acknowledgements

We are indebted to all members of the laboratories in Penzberg for valuable discussions and Prof. Dr. Oliver Eickelberg for manuscript proofreading. We thank Attila Csordas and the other members of the PRIDE team at the EMBL-EBI for their assistance in making the data used for this manuscript publicly accessible via the PRIDE repository. COBAS, COBAS E and COBAS INTEGRA are trademarks of Roche.

Author Contributions

Conceived and designed the experiments: KB MW PB. Performed the experiments: KB KD MW AL VR LA KK GE US. Analyzed the data: KB KD MM PB. Contributed reagents/materials/analysis tools: PB HW DR. Wrote the manuscript: KB MM PB.

References

1. Briggs J, Panfili PR (1991) Quantiﬁcation of DNA and protein impurities in biopharmaceuticals. Anal Chem 63: 850-859. doi:10.1021/ac00009a003. PubMed: 1858980.
2. Janeway CA, Travers P, Walport M, Shlomchik M (2005) Immunobiology: the immune system in health and disease. Garland Science Publishing, New York, USA p. 560 pp. 90-91
3. Wang X, Hunter AK, Mozier NM (2009) Host cell proteins in biologics development: Identification, quantitation and risk assessment. Biotechnol Bioeng 103: 446-458. doi:10.1002/bit.22304. PubMed: 19388135.
4. Gao SX, Zhang Y, Stansberry-Perkins K, Boko A, Bai S et al. (2011) Fragmentation of a highly purified monoclonal antibody attributed to residual CHO cell protease activity. Biotechnol Bioeng 108: 977-982. doi:10.1002/bit.22982. PubMed: 21404269.
5. ICH (1999) Guidance for industry Q6B specifications: Test procedures and acceptance criteria for biotechnological/bioloigical products. Rockville, MD, U.S. Dept. of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research.
6. Leader B, Baca OJ, Golan DE (2008) Protein therapeutics: a summary and pharmacological classification. Nat Rev Drug Discov 7: 21-39. doi: 10.1038/nrd2399. PubMed: 18097456.
7. Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V et al. (1997) The complete genome sequence of Escherichia coli K-12. Science 277: 1453-1462. doi:10.1126/science.277.5331.1453. PubMed: 9278503.
8. Gibbons RA, Weinstock GM, Metzker ML, Muzny DM, Sodergren EJ et al. (2004) Genome sequence of the Brown Norway rat yields insights into mammalian evolution. Nature 428: 493-521. doi:10.1038/nature02426. PubMed: 15057822.
9. Mouse Genome Sequencing Consortium, Waterston RH, Lindblad-Toh K, Birney E, Rogers J et al. (2001) Initial sequencing and comparative analysis of the mouse genome. Nature 420: 520-562. doi:10.1038/ nature01262. PubMed: 12466850.
10. Hart RA, Rinas U, Bailey JE (1990) Protein composition of Vitreoscilla hemoglobin inclusion bodies produced in Escherichia coli. J Biol Chem 265: 12728-12733. PubMed: 2197280.
11. Rinas U, Bailey JE (1992) Protein compositional analysis of inclusion bodies produced in recombinant Escherichia coli. Appl Microbiol Biotechnol 37: 609-614. PubMed: 1369400.

12. Rinas U, Boone TC, Bailey JE (1993) Characterization of inclusion bodies in recombinant Escherichia coli producing high levels of porcine somatotropin. J Biotechnol 28: 313-320. doi: 10.1016/0168-1656(93)90179-Q. PubMed: 7765564.

13. Veeraragavan K (1989) Studies on two major contaminating proteins of the cytoplasmic inclusion bodies in Escherichia coli. FEMS Microbiol Lett 52: 149-152. PubMed: 2696277.

14. Eaton LC (1995) Host cell contaminant protein assay development for recombinant biopharmaceuticals. J Chromatogr A 705: 105-114. doi: 10.1016/0021-9673(94)01249-E. PubMed: 7620564.

15. Hoffman K (2000) Strategies for host cell protein analysis 13. Biopharm. pp. 38-45.

16. Rathore AS, Sobacke SE, Kocot TJ, Morgan DR, Dufield RL et al. (2003) Analysis for residual host cell proteins and DNA in process streams of a recombinant protein product expressed in Escherichia coli cells. J Pharm Biomed Anal 32: 1199-1211. doi: 10.1016/S0731-7085(03)00157-2. PubMed: 12907264.

17. Savino E, Hu B, Sellers J, Sobjak A, Majewski N et al. (2011) Development of an in-house, process-specific ELISA for detecting HCP in a therapeutic antibody, Part 2. Bioprocess International 9: 69-75.

18. Wang X, Schommogy T, Wells K, Mozier NM (2010) Improved HCP quantitation by minimizing antibody cross-reactivity to target proteins. Bioprocess International 105: 306-316. doi:10.1002/bit.22532. PubMed: 19739084.

19. Wiencek R, Fowler PA (2003) Mass spectrometry-based proteomics. Nature 422: 198-207. doi:10.1038/nature01511. PubMed: 12634793.

20. Issaq HJ, Chan KC, Janini GM, Conrads TP, Veenstra TD (2005) Multidimensional separation of peptides for effective proteomic analysis. J Chromatogr B Analyt Technol Biomed Life Sci 817: 35-47. doi:10.1016/j.chromb.2004.08.019. PubMed: 1568075.

21. Motoyama A, Yates JR 3rd (2008) Multidimensional LC separations in shotgun proteomics. Anal Chem 80: 7187-7193. doi:10.1021/ac8013669. PubMed: 18826178.

22. Raschbaur MP, Wolters D, Yates JR 3rd (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol 19: 242-247. doi:10.1038/85686. PubMed: 11231557.

23. Oneanu CE, Xenopoulos A, Fadgen K, Murphy J, Skilton SJ et al. (2012) Analysis of host-cell proteins in biotherapeutic proteins by comprehensive online two-dimensional liquid chromatography/mass spectrometry. mAbs 4: 24-44.

24. Schenauer MR, Flynn GC, Goetze AM (2012) Identification and quantification of host cell protein impurities in bioteherapeutics using mass spectrometry. Anal Biochem 428: 150-157. doi:10.1016/j.ab.2012.05.018. PubMed: 22640604.

25. Kim EE, Wyckoff HW (1991) Reaction mechanism of alkaline phosphatase based on crystal structures. Two-metal ion catalysis. J Mol Biol 218: 449-464. doi:10.1016/0022-2836(91)90724-K. PubMed: 2010919.

26. Sowadski JM, Handschumacher MD, Murhy HM, Foster BA, Wyckoff HW (1985) Refined structure of alkaline phosphatase from Escherichia coli at 2.8 A resolution. J Mol Biol 186: 417-433. doi:10.1016/0022-2836(85)90179-Q. PubMed: 7763564.

27. Cha MK, Kim HK, Kim IH (1995) Thioester-linked “thiol peroxidase” from periplasmic space of Escherichia coli. J Biol Chem 270: 28635-28641. doi:10.1074/jbc.270.48.28635. PubMed: 7493831.

28. Brune M, Schumann R, Wittinghofer F (1985) Cloning and sequencing of the adenylate kinase gene (adk) from Escherichia coli. Nucleic Acids Res 13: 7139-7151. doi:10.1093/nar/13.19.7139. PubMed: 2907739.

29. Kellf F, Petrella S, Mercuri F, Sauvage E, Herman R et al. (2010) Specific structural features of the N-acetyluramoyl-L-alanine amide Amid from Escherichia coli and mechanistic implications for enzymes of this family. J Mol Biol 397: 249-259. doi:10.1016/j.jmb.2009.12.038. PubMed: 20036252.

30. Steinj J, Schlichting I, Wittinghofer A (1990) Structurally and catalytically important residues in the phosphate binding loop of adenylyl kinase of Escherichia coli. Biochemistry 29: 7451-7459. doi:10.1021/bi00484a014. PubMed: 22223776.

31. Talarico TL, Dev IK, Dallas WS, Ferone R, Ray PH (1991) Purification and partial characterization of 7,8-dihydro-6-hydroxymethylpterin- pyrophosphokinase and 7,8-dihydropteroate synthase from Escherichia coli MC4100. J Bacteriol 173: 7029-7032. doi:10.1128/JB.173.21.7029-7032. PubMed: 1657875.

32. Vizcaino JA, Côté RG, Csordas A, Dianes JA, Fabregat A et al. (2013) The PRoteomics IDENTifications (PRIDE) database and associated tools: status in 2013. Nucleic Acids Res 41: D1083-D1089. doi:10.1093/nar/gks1262. PubMed: 23203882.

33. Wang R, Fabregat D, Rios D, Ovelleiro D, Foster JM et al. (2012) PRIDE Inspector: a tool to visualize and validate MS proteomics data. Nat Biotechnol 30: 135-137. doi:10.1038/nbt.2112. PubMed: 22318026.