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Capacitive immunosensor for the detection of host cell proteins

Kosin Teeparuksapun[a,b,c], Martin Hedström[a], Proespichaya Kanatharana[b,c], Panote Thavarungkul[b,d], Bo Mattiasson[a,*]

[a] Department of Biotechnology, Lund University, Box 124, S-22100 Lund, Sweden
[b] Trace Analysis and Biosensor Research Center (TAB), Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[c] Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[d] Department of Physics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

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ABSTRACT

A new analysis for monitoring host cell proteins in preparations of transgenically produced protein pharmaceuticals is described. A capacitive biosensor with a very high sensitivity is used to monitor trace amounts of host cell proteins. The sensor consists of a gold electrode, the surface of which is well insulated and on which a preparation of a population of polyclonal antibodies raised against the complete protein set-up of the host cell are immobilized.

Host cell proteins are present at very low concentrations during the production of a transgenic protein. The system studied here is a model system with an enzyme expressed in *Escherichia coli* (*E. coli*). Due to the high sensitivity, it may even be possible to dilute the samples to be analyzed, thereby reducing a negative influence from non-specific binding to the sensor surface.

1. Introduction

Gene technology has made it possible to transfer genes from one organism to another and to express the gene product in the new host cell. This development has led to dramatic change in the production conditions of, e.g. pharmaceutical proteins. The development has gone from an earlier hard struggle to isolate compounds from large amounts of biomass where the target protein was only a trace component into situations when the cloned product might constitute up to 10% or more of the total protein content. The purification protocols have also been streamlined and they give higher over-all yields than before (Gyrolab Bioaffy, 2009; Anicetti and Hancock, 1994; World Health Organization, 1991).

In parallel to this development, there have also been improvements in the ability to detect trace impurities, i.e. contaminating compounds present at very low concentrations (O'Keefe et al., 1993).

Process monitoring in the downstream area has so far, to a large extent, been focused on the monitoring of target molecules and less on the impurities. However, there are several substances or groups of substances that are of interest to quantify during the process (DiPaolo et al., 1999). Examples on such compounds are endotoxins, DNA, host cell proteins (HCP), i.e. proteins from the host cell where the foreign gene was expressed, protein ligands from affinity steps, etc. The concentration of these compounds will by necessity be low and therefore very sensitive assay methods are needed. The sensitivity demands put limitations on the analytical protocols used. Immuno-based sensors have, after a slow start (Mattiasson, 1984; Berggren and Johansson, 1997; Gizeli and Lowe, 1996), gained in popularity, and represent today a realistic analytical alternative. The studies reported here are based on results obtained using a capacitive immunosensor (Berggren et al., 1998; Hedström et al., 2005; Limbut et al., 2006).

In this report we present a novel procedure for the monitoring of host cell proteins from a process where *Escherichia coli* (*E. coli*) was the production strain. Since a broad spectrum of proteins can be possible contaminants, it is required to use a polyclonal antibody preparation raised against the total proteome of the host cell.

2. Materials and methods

2.1. Reagents and solutions

Polyclonal antibodies raised against a standard *E. coli* proteome (anti-HCP) were a kind gift from Pfizer (St. Louis, USA). HCP (*E. coli*) came from Novozymes Biopharma (Lund, Sweden). Human serum albumin (HSA) was obtained from Dako (Denmark). Bovine serum albumin (BSA), thioctic acid and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) were obtained from Sigma

* Corresponding author. Tel.: +46 462228264; fax: +46 462224713.
E-mail address: bo.mattiasson@biotek.lu.se (B. Mattiasson).

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(St. Louis, USA), 1-dodecanethiol was obtained from Aldrich (Deisenhofen, Germany). All other chemicals were of analytical grade. Glass microscope slide (25.4 mm × 76.2 mm) (Sailbrand 23, China) was used as the base for electrodes fabrication using D.C. sputtering (NECTEC pilot plan, Thailand). All buffers were prepared with water obtained from a Milli-Q system. Before use, all buffers were filtered through a Millipore filter with pore size 0.22 μm with subsequent degassing.

2.2. Immobilization of anti-HCP

Sputtered gold electrodes were plasma cleaned (Mod. PDC-3XG, Harrich, NY) for 20 min. The cleaned electrodes were immediately put in thioctic solution (2%, w/w in ethanol) for 24 h. In this step a self-assembled monolayer (SAM) was formed on the gold surface. The electrodes were thoroughly rinsed with ethanol to remove unbound thioctic acid and thereafter dried under vacuum. Subsequently, the gold electrodes were put in EDC solution (1%, w/w in dry acetonitrile) for 5 h where a terminal OH group of the thiol was activated to form *O*-acylisourea (Hedström et al., 2005). The electrodes were then rinsed with 100 mM sodium phosphate buffer, pH 7.0 and dried under nitrogen gas. The electrodes were then immersed in anti-HCP (35 μg/mL) in 100 mM sodium phosphate buffer, pH 7.0 overnight at 4 °C. The terminal amino groups of antibodies displace the *O*-acylisourea and thus bind covalently to the monolayer. As a last step in the preparation, the electrodes were placed in 10 mM 1-dodecanethiol ethanolic solution for 20 min in order to cover bare parts of the gold surface. When not in use electrodes were kept at 4 °C in a closed Petri dish filled with nitrogen gas.

2.3. Capacitive measurement

The capacitive measurements were done through a potentiostatic method, similar to the one described by Hedström et al. (2005). However, instead of using solid gold rods as working electrodes, sputtered gold electrodes were used. Fig. 1 shows the schematic diagram of the flow injection capacitive immunosensor system. Discrete pulse injections of samples with a concentration interval 1.0×10^{-20} to 1.0×10^{-11} M of HCP were performed using a sample injector with a 250 μL loop. The flow rate of the carrier buffer (10 mM sodium phosphate pH 7.0) was 100 μL/min. A regeneration step with 25 mM glycine–HCl, pH 2.5 was performed between each analyte injection.

2.3.1. Reproducibility

HCP was detected repeatedly, using an assay cycle involving binding, measurement, regeneration and reconstitution intermittently over 2 days (14 times/day). The reproducibility of the anti-HCP modified electrodes was evaluated by monitoring the change of capacitance signal at the same concentration of standard HCP 1.0×10^{-15} M.

2.3.2. Specificity of the anti-HCP electrode

The effect of other substances that might interfere with the response to HCP in the capacitive immunosensor system was studied. The selectivity was investigated by using HSA and BSA as model proteins. The protein samples (HCP, HSA and BSA) were injected in triplicates at the same concentration range (1.0×10^{-15} to 1.0×10^{-13} M).

2.4. Purification of lactate dehydrogenase

In this study, the production of lactate dehydrogenase (LDH) was investigated as a model system. *E. coli* was used as a host for the production of LDH and the residual HCP impurities during purification processes and in final purified protein were investigated. The preparation of supernatant sample from cultivated *E. coli* cells for LDH production is shown in Fig. 2.

E. coli was grown in Luria-Bertani (LB) broth (20 g/L) (Difo™, Lennox). The culture was agitated at 180 rpm for 24 h at 30 °C. The cells were then centrifuged at 2550 × *g* for 30 min. One milliliter of supernatant was collected and labelled as S_1. The cell pellets

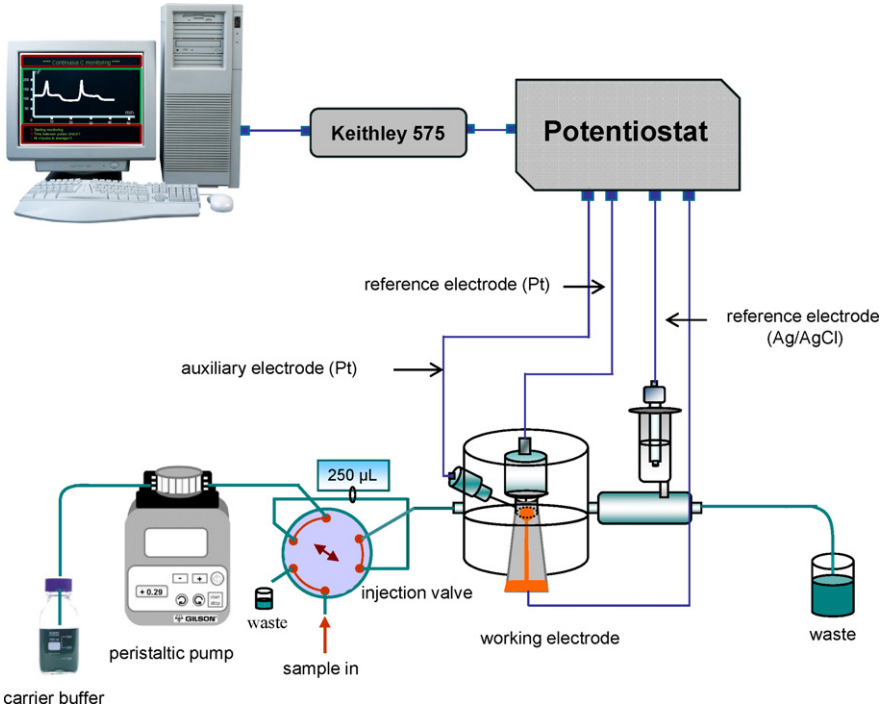

Fig. 1. Schematic diagram showing the flow injection capacitive immunosensor system.

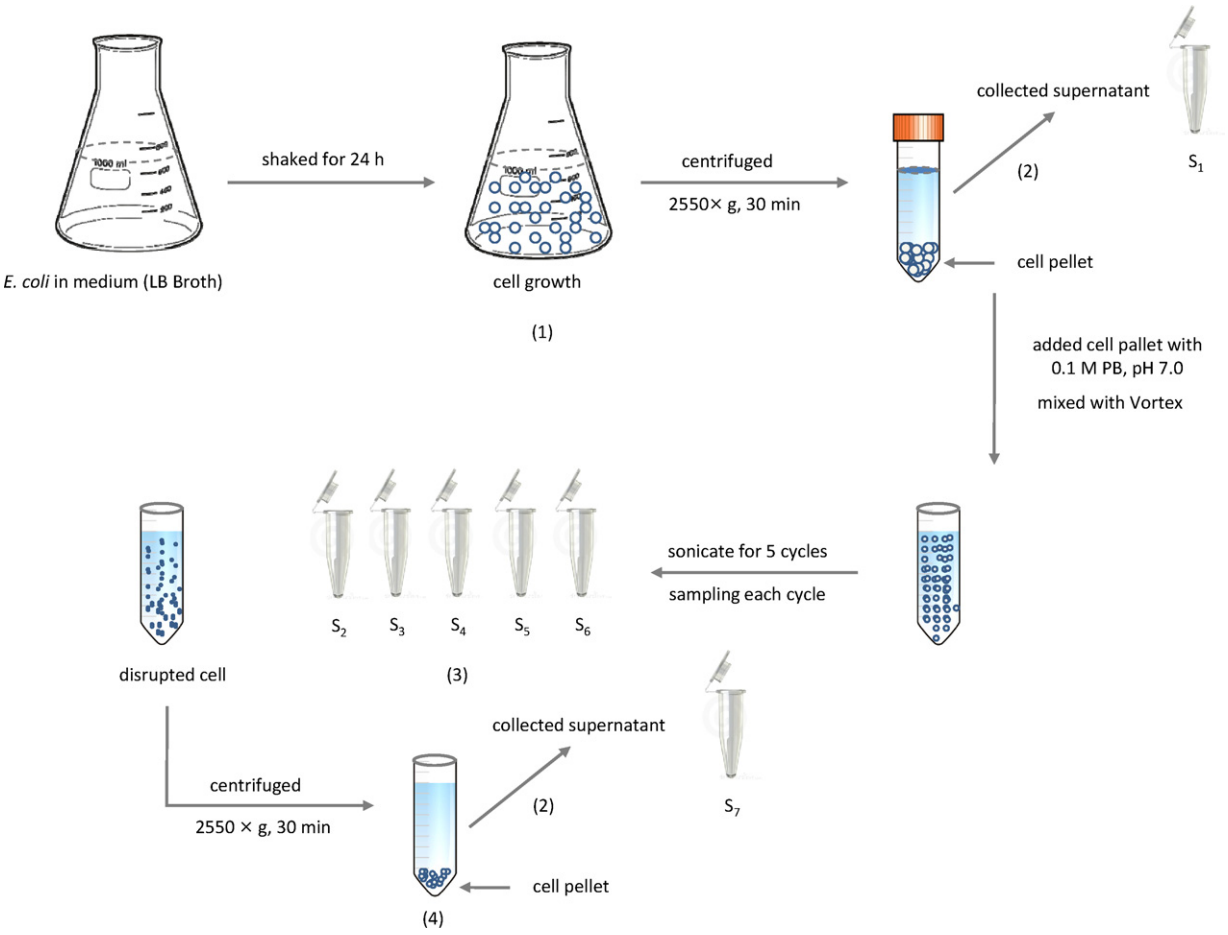

Fig. 2. Schematic diagram of the *E. coli* preparations for the purification of LDH. (1) Fermentation of cells in LB broth medium, (2) collection of supernatant (S_1), (3) collection of samples in each sonication cycle (S_2–S_6) and (4) collecting supernatant of the disrupted cell suspension after sonication (S_7).

were re-suspended in 20 mL of 0.1 M phosphate buffer, pH 7.0 and disruption to release LDH was accomplished by sonication using a probe at an amplitude of 60 for 1 min. Here, 1 mL of sample was retrieved with 30-s intervals and the sample collection was repeated 5 times (S_2–S_6). The cell lysate was centrifuged at 2550 × *g* for 30 min and a 1 mL sample (S_7) was collected and stored at 4 °C. The supernatant was used for purification with immobilized metal affinity chromatography (IMAC). The sample (S_7) were kept for analysis of total protein content (bicinchoninic acid (BCA)), LDH activity (enzymatic assay, NADH/NAD) and the presence of HCP (capacitive immunosensor).

2.4.1. Immobilized metal affinity chromatography

Sepharose CL-6B with immobilized iminodiacetic acid (IDA) was packed in a column with the dimensions 8 × 10 mm. A solution of 5 mg/mL $CuSO_4$ was pumped through the column in order to saturate the chelating groups with Cu^{2+} ions. The column, which was operated at 0.5 mL/min, was equilibrated with 20 mL of a 20 mM Tris–HCl buffer (pH 7.3) containing 10 mM imidazole and 0.5 M NaCl to remove unbound Cu^{2+}. The supernatant from the centrifugation step (6 mL) was loaded onto the column, which was then washed with Tris–HCl buffer. Fractions collected from the washing step were stored at 4 °C for further analysis. During the washing step, the absorbance at 280 nm was measured until the value was close to zero. His_6-tag protein of LDH which bound to the Cu-IDA with high affinity was then eluted with 200 mM imidazole. Fractions during the eluting step were collected in the same way as described above.

2.4.2. Total protein analysis

The total protein content was determined using the BCA assay. BCA working reagent (1:50 ratio of $CuSO_4$ to BCA, 220 μL) was added to 0.01 mL of BSA protein standard (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL), blank and purified sample solutions in a 96-well plate. After incubating the plate at 37 °C for 30 min, the absorbance of each solution was measured at 562 nm.

2.4.3. LDH activity assay

The LDH activity of the samples from the production process was assayed spectrophotometrically by studying the reaction rate for the enzymatic oxidation of NADH at 340 nm according to Eq. (1).

$$\text{NADH} + \text{pyruvate} + \text{H}^+ \overset{\text{LDH}}{\longleftrightarrow} \text{lactate} + \text{NAD}^+ \quad (1)$$

The assay was performed by mixing 2.8 mL of 0.2 M Tris–HCl, pH 7.3 with 0.05 mL of 6.6 mM NADH and 0.05 mL of 30 mM sodium pyruvate. The reaction was initiated by adding 50 μL of diluted LDH samples and ΔA_{340}/min was recorded.

2.5. Analysis of HCP using the capacitive immunosensor

The purification of LDH using IMAC was used as a model technique for investigating the presence of HCP in different stages of the production process. Samples were analyzed for HCP using the capacitive immunosensor system described schematically in Fig. 1. Standard HCP solutions with concentrations from 1.0×10^{-17} to 1.0×10^{-13} M were injected in triplicate

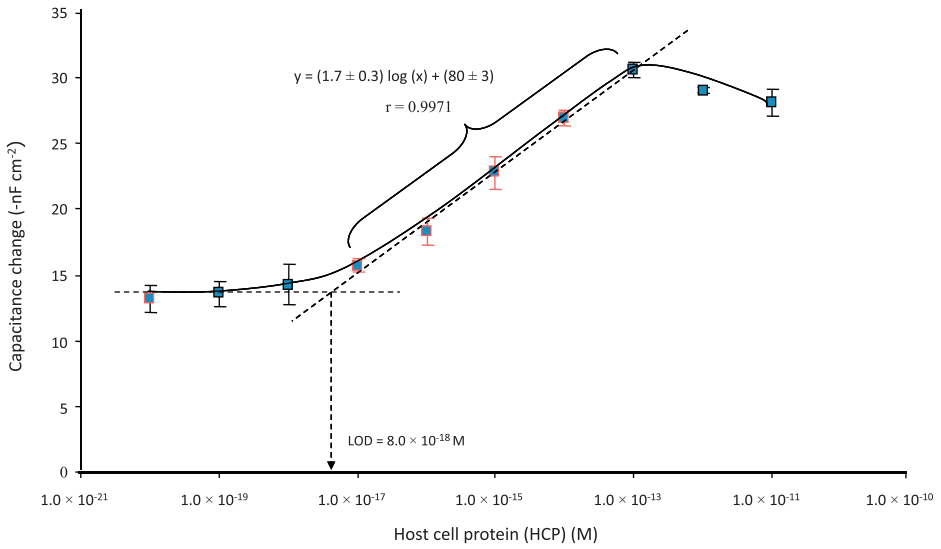

Fig. 3. Capacitance change ($-$nF cm^{-2}) versus logarithm of the HCP concentrations (the response from the blank solution (injected running buffer) was 5.2 ± 0.3 $-$nF cm^{-2}).

and used to perform the calibration curve. The concentrations of HCP in the unknown samples were then estimated ($n = 1$) from the calibration curve made from the assays of standard solutions.

3. Results

3.1. Capacitive measurement

Capacitive measurements were performed using the potentiostatic method. Standard solutions of HCP in the concentration range of 1.0×10^{-20} to 1.0×10^{-11} M were injected to the capacitive immunosensor system in order to investigate the sensitivity, linearity and limit of detection. The graph from this study was obtained by plotting the capacitance change ($-$nF cm^{-2}) versus the logarithm of HCP molar concentration (see Fig. 3). A linear relationship was obtained between 1.0×10^{-17} and 1.0×10^{-13} M and the limit of detection was determined to be 8.0×10^{-18} M, based on IUPAC recommendation (Buck and Lindner, 1994).

3.1.1. Reproducibility

The anti-HCP modified electrodes were evaluated in terms of reproducibility by monitoring the capacitance change ($-$nF cm^{-2}) at the same concentration of standard HCP (1.0×10^{-15} M), 14 times/day for 2 days. After each analysis, 250 μL of 25 mM glycine–HCl pH 2.5 was injected to break the bonds between the antibodies and HCP. The percentage residual activity of the anti-HCP sensors versus the number of injections is shown in Fig. 4. During the first 25 cycles, the binding activity of anti-HCP retained about 98 ± 5% of the original capacitance change.

Hence, the anti-HCP electrodes can be reused with good reproducibility up to 25 times with an acceptable relative standard deviation (RSD) of ≤5%. After 25 times of regeneration the binding activity of the electrode decreased rapidly. The reduction of the activity may be caused by the loss of antibodies from the surface, or perhaps the loss of SAM layer. However, it was reported that the immobilization using SAM is very strong and was not lost after several regeneration cycles (Thavarungkul et al., 2007). Another more trivial explanation might be microbial infection of the assay system (Borrebaeck et al., 1978). This might have happened since the assay procedure was not done under sterile conditions and with a buffer without added bactericidal compounds. Similar rapid loss of activity of immobilized antibodies has been reported earlier in connection to stability studies of immunochemical binding assays (Limbut et al., 2006).

3.1.2. Selectivity

An assay with high sensitivity may also be subjected to pronounced disturbances due to non-specific binding from other macromolecules in the sample. In this case HSA and BSA represented proteins that might cause non-specific binding and therefore affect the assay.

Fig. 5 shows the response of HCP, HSA, and BSA over the same concentration range from 1.0×10^{-15} to 1.0×10^{-13} M. The capacitance change ($-$nF cm^{-2}) obtained from injections of HCP was significantly higher than the two obtained from HSA and BSA. In addition, the decrease in capacitance change related to HCP injections corresponded well to the concentration stated for the standard solution (1.0×10^{-15} to 1.0×10^{-13} M of HCP). In contrast, the capacitance changes of HSA and BSA at every concentration were almost the same. However, at 1.0×10^{-15} M the signal responses obtained from HSA and BSA were close to that of HCP but also higher than the response from the injection of blank (4.3 ± 0.2 $-$nF cm^{-2}). These results indicate that one needs to give non-specific interactions more attention in future work. Work is in progress in this direction.

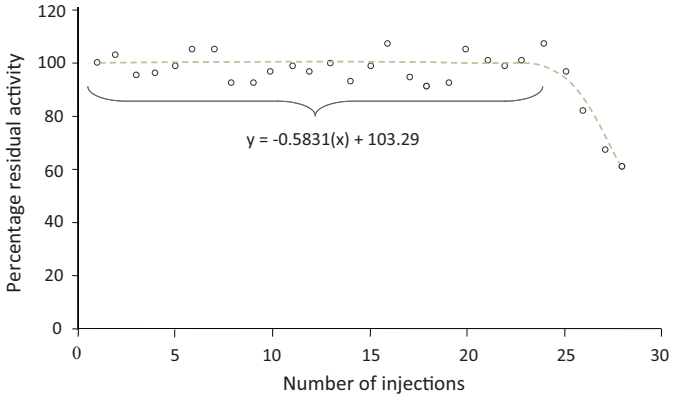

Fig. 4. Reproducibility of the response from an anti-HCP modified electrode to injections of 250 μL of a standard solution of HCP antigen (1.0×10^{-15} M) with regeneration and reconditioning steps between each individual assay.

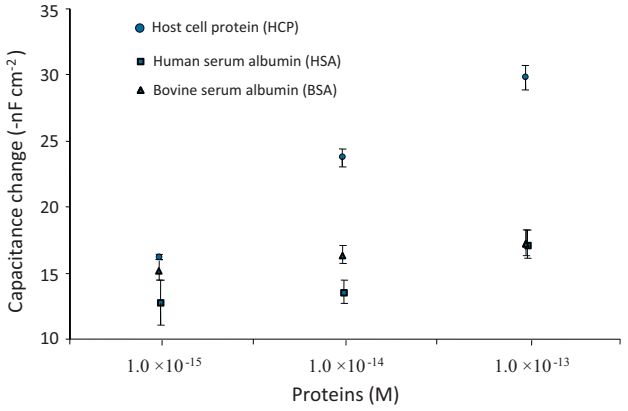

Fig. 5. Effect of anti-HCP to HCP, HSA and BSA.

In this experiment, each protein was injected in triplicate, which amounts to 27 injections. The last three injections relate to BSA at a concentration of 1.0×10^{-13} M. Unlikely the results in the reproducibility study (see Section 3.1.1) the capacitance change after 25 injections are not decreased dramatically and give similar responses to the previous injection. This result supported the fact that the rapid decrease in capacitance signal observed in the reproducibility study is more likely due to microbial infection as explained previously.

3.2. Purification of LDH

E. coli was used as a host for the production of LDH. The purification of LDH was used as a model system in order to investigate the residual HCP impurities during the purification processes and in final purified protein. The samples after cell disruption (S_7) and sample after purification using IMAC were tested for the activity of LDH, the total protein content and residual HCP impurities.

3.2.1. Immobilized metal affinity chromatography

The supernatant after cell disruption (S_7) was used for the purification of LDH using IMAC. The absorbance at 280 nm of the eluate during the washing steps is shown in Fig. 6. Other impurities, e.g. proteins lacking the His_6-tag or without surface-exposed His-residues will consequently not bind to the Cu-IDA and hence be washed out. Most of these unbound proteins were found in fraction 2 ($A = 1.96$) with minor amounts in further fractions. After 20 fractions of the washing step, the absorbance at 280 nm approached zero. The target LDH protein bound to the Cu-IDA was then eluted with a pulse of 200 mM imidazole.

3.2.2. Total protein analysis

The protein concentration of each sample was assayed. The concentrations of total protein increased from 6.11 to 8.56 mg/mL with the cell disruption cycles (S_2–S_5). For sample S_6, the protein concentration was 8.1 mg/mL which is slightly lower than that of the earlier cycle (S_5, 8.6 mg/mL). The decrease in protein content might be caused by the denaturation of some proteins during cell disruption due to the elevated temperature in the solution during sonication. The samples from the sonication cycles (S_2–S_6) were pooled and centrifuged to obtain the supernatant (S_7). The protein concentration in this solution was determined to 7.7 mg/mL as shown in Fig. 7A.

3.2.3. Assay for LDH activity

Fractions obtained from the sonication step together with the washing and eluting steps from the IMAC purification process were assayed for the LDH activity. The cell-free culture filtrated from the cultivation (S_1), did as expected not contain any LDH activity. However, in the cell disruption step (S_2–S_6), where the cells were lysed the LDH concentration increased from 1.9 to 2.6 μg/mL (samples S_2–S_4), whereas the LDH concentrations of cycles S_5–S_6 slightly decreased (2.5 μg/mL). For sample S_7 (pooled mixture of S_2–S_6) the LDH concentration was determined to 2.2 μg/mL (Fig. 7B). Furthermore, no LDH activity could be detected in the samples from the fractions related to the IMAC washing peak. However, in the eluting step (E_{25}) the enzyme activity was detected with a total concentration of 0.5 μg/mL and a total volume of 6 mL.

3.3. HCP-analysis using the capacitive biosensor

In this study, the purification of LDH using IMAC was used as a model study for the investigation of residual HCP impurities in the purification process. The analysis was performed using a capacitive immunosensor system. The samples from the first collected supernatant, S_1 and cell disruption step, S_2–S_6, were diluted 10 and

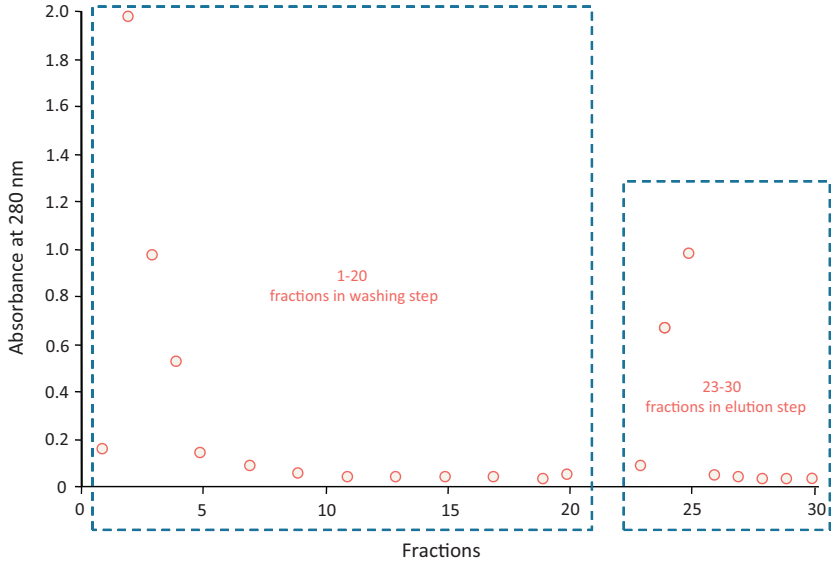

Fig. 6. Absorbance (280 nm) of fractions obtained from washing and eluting steps in the purification of LDH from *E. coli* using IMAC, (1–20 and 23–30 are fractions from the washing and eluting steps, respectively).

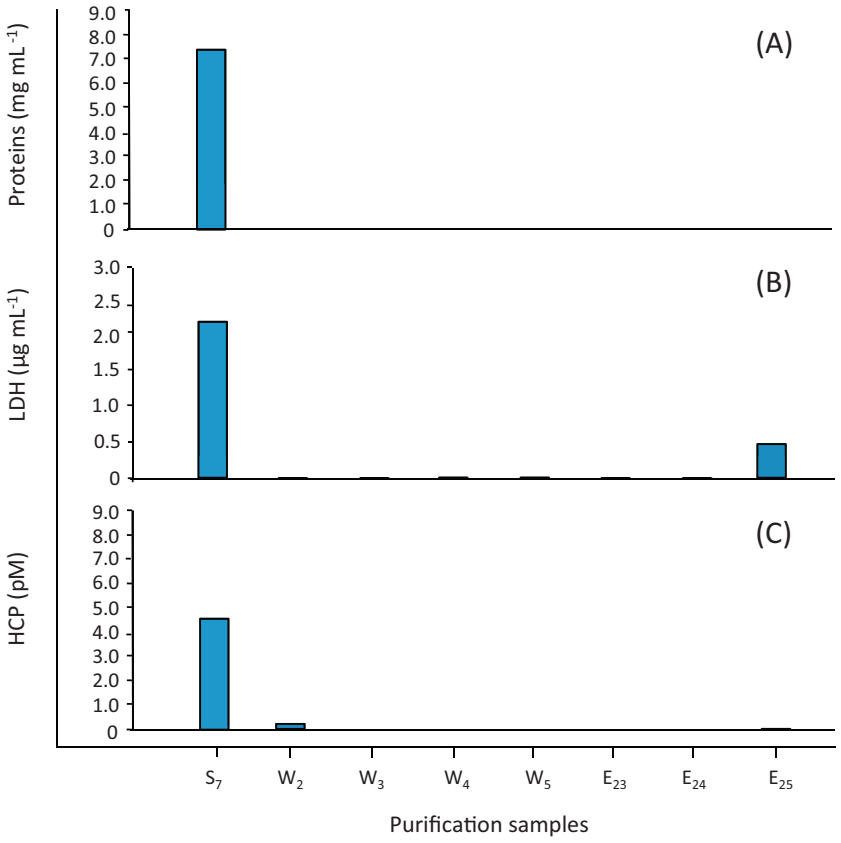

Fig. 7. (A) Concentration of total protein for the supernatant (S_7) ($n = 2$). In (B) the concentrations of LDH obtained from the pool of the samples after sonication of cells and removal of cell debris (S_7) and samples during purification with IMAC; washing (W_2–W_5) and eluting (E_{23}–E_{25}) steps ($n = 2$). In (C) is shown the concentration of HCP in samples obtained from the purification process of LDH produced recombinantly in *E. coli*. The pooled fractions (S_7), samples during purification using IMAC; washing (W_2) and eluting (E_{25}) steps ($n = 1$).

100 times, respectively, and injected to the capacitive immunosensor system. The capacitive signals ($-$nF cm^{-2}) were compared to a calibration curve generated from standard solutions of HCP with known concentration of the target. Again, no HCP could be detected in sample S_1 while the concentration of HCP in sample S_2 was determined to be 2.4×10^{-12} M. Further disruption resulted in the release of more HCP (S_3, 8.3×10^{-12} M). The concentration of HCP was decreased for the third cycle ($S_4 = 2.5 \times 10^{-12}$ M) and fourth cycle ($S_5 = 1.2 \times 10^{-12}$ M). After the fifth cycle (S_6) the concentration of HCP was measured to be 1.4×10^{-12} M. When samples S_2–S_6 were pooled and then centrifuged at $2550 \times g$ (S_7), the concentration of HCP was 4.4×10^{-12} M (Fig. 7C). From this study, we can investigate the presence of HCP released from the cell during disruption steps.

As shown in Fig. 7C, the analysis of HCP using the capacitive immunosensor was also performed on the samples retrieved from the IMAC purification. The concentration of HCP found in the fraction from washing step (W_2) was 5.3×10^{-14} M whereas analysis of the fractions from the elution step showed that the amount of the HCP contaminant was below the limit of the detection for the capacitive sensor.

4. Conclusion

The capacitive immunosensor was proven effective for the task of determining the concentration profile of HCP during recombinant *E. coli* production; cultivation as well as the downstream process.

To monitor host cell proteins is an interesting challenge and it represents some aspects not seen when tracing a specific compound (Shukla et al., 2008). The host will release a range of different structures to the cultivation medium, and it is important to monitor them as an integrated measure. It might be tempting to use one or two reporter proteins and base the analysis on them. However, if during the downstream processing, there is an uneven level of elimination of impurities, then a "reporter" protein may not be representative any longer. Thus, the integrated approach is to prefer. The impurities that will be most difficult to eliminate are those that have properties similar to those of the target protein. This means that a protein that is present at very low concentrations in the starting material may be concentrated along the separation sequence and eventually become the dominating host cell protein species.

Furthermore, direct monitoring of binding between immobilized ligands and host cell proteins is to prefer rather than using labelled reagents. Also here one avoids many of the problems with regard to abundance of individual protein species in the labelled preparation. This is of course important for the same reasons as given above concerning avoidance of reporter-proteins.

The ligands to use in this connection need to be polyclonal antibodies raised against a full collection of host cell proteins, e.g. a cell homogenate. Monoclonal antibodies will not be desirable here, since one needs to use a cocktail of many different antibodies. The same limitations appear here as discussed above concerning use of reporter proteins. It is however important to realize that there is a risk when using a polyclonal preparation since then the proportion of antibodies raised against the different protein components jointly called host cell proteins is not defined. This may lead to situations when a rare protein in the immunization mixture gives rise to a small amount of antibodies, while during the downstream

processing of the target protein this rare protein becomes a more important component of the total host cell protein burden. There might be a risk of lack of binding sites on the sensor and then the signal will be lower than what otherwise would have been the case. To address this challenge it is recommended to use dilutions of the sample to be analyzed, since upon such analyses it will become obvious if there is an uneven level of binding of the host cell proteins to the sensor.

The system discussed here thus represents an interesting challenge from an analytical point of view and the use of direct monitoring of binding to a population of polyclonal antibodies seems to meet most of the demands. There will be a need for a battery of host cell protein analyses since each cell type or even each strain needs to be monitored with a set of antibodies specially raised against the proteins of that specific host.

Acknowledgement

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