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Measurement of porcine haptoglobin in meat juice using surface acoustic wave biosensor technology

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ABSTRACT

Aim of the study was the application of biosensor technique to measure the concentration of an acute phase protein (APP) within complex matrices from animal origin. For the first time, acute phase protein haptoglobin (Hp) was detected from unpurified meat juice of slaughter pigs by a label-free biosensor-system, the SAW-based sam®5 system. The system uses a sensor chip with specific antibodies to catch Hp while the mass-related phase shift is measured. The concentration is calculated as a function of these measured phase shifts. The results correlate very well with reference measurement results obtained by enzyme-linked immunosorbent assay (ELISA), $R = 0.98$. The robust setup of the surface acoustic wave (SAW)-based system and its ability to measure within very short time periods qualifies it for large-scale analyses and is apt to identify rapidly pigs in the meat production process whose consumption would have an increased risk for consumers.

1. Introduction

Whenever the tissue of a pig is damaged, e.g. due to inflammation or bacterial infection, the animal shows an unspecific immune response, the acute phase reaction (Gruys, Toussaint, Niewold, & Koopmans, 2005). Within 6 to 48 h, the production of more than 30 different acute phase proteins (APP) increases (Miller, Wait, Sipos, & Gemeiner, 2009). One of these APPs is haptoglobin (Hp) (Baumann & Gauldie, 1994). A role of the blood plasma protein Hp is to bind and transport hemoglobin (Murata, Shimada, & Yoshioka, 2003). Porcine Hp has a molecular weight of about 120 kDa. The α2-globulin consists of two light α-chains (9.1 kDa) and two heavy β-chains (40 kDa) (Petersen, Nielsen, & Heegaard, 2004). It is not exclusively present at inflammation, but it increases significantly from a base level by more than ten times (Piñeiro, Gymnich, Knura, Piñeiro, & Petersen, 2009). Since the half-life of Hp is about four days, an increase is still detectable after several days (Hall, Eurell, Hansen, & Herr, 1992). Hp can be used as a parameter of infection and inflammation (Eckersall, 2000). Thus, Hp is an excellent not disease-specific marker for health assessment, e.g. of pigs in a screening test (Gymnich, 2001; Knura-Deszczka, 2000; Murata et al., 2003; Petersen, Dideriksen, Christiansen, & Nielsen, 2002; Petersen et al., 2004; Piñeiro, Gymnich, et al., 2009; Piñeiro, Piñeiro, et al., 2009). The evaluation of animal health and welfare has also been investigated based on Hp concentrations in meat juice (Hiss et al., 2003; Piñeiro, Lampreave, & Alava, 2009; Witten, 2006).

Many methods have been developed to determine Hp from various origins. Manual methods detecting Hp content are EIA/ELISA outlined by Lequin (2005). Hp from porcine origin has also been detected by enzyme-linked immunosorbent assay (ELISA) (Hiss et al., 2003). Another assay measuring Hp in the samples of various body fluids of swine is based on time-resolved immunofluorometry (TR-IFM) (Gutiérrez, Martínez-Subiela, & Cerón, 2009). Lately, (semi-) automated methods to measure APPs have been developed, i.e. immunoturbidimetric assays (Saco, Fraile, Giménez, Canalias, & Bassolos, 2010) and automated spectrophotometric methods (Martinez-Subliela, Tecles, & Cerón, 2007). At present, APPs mostly are quantified by ELISA.

Tecles et al. (2007) presented a comparison of different commercial APP tests. Intra and inter assay coefficients of variation (CV) of the Hp assay were lower than 5.7% (see Table 1) at any tested APP concentration. Dilutions of samples with high concentrations resulted in linear regression equations with coefficients of correlation (R) ranging from 0.98 to 0.99. The detection limit (0.02 g/L) of the Hp assay was low enough to detect Hp levels even in healthy animals (Tecles et al., 2007). Table 1 gives examples of intra and inter assay CV for different methods that are used to measure acute phase protein in serum of pigs.

Although the results of ELISA within one laboratory are trustable, these measurements still are tedious and time consuming (Skinner, 2001). Specialized laboratory equipment is necessary as well as technical training of the laboratory personal. Therefore, the pig is already

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Table 1
Intra- and inter-assay coefficients of variation (CV) due to different measurement methods of porcine haptoglobin.

Method	Protein	Mean (SD) [g/L]	CV [%]		Author
ELISA	Hp	0.86 (0,03)	4.00	Intra-assay	Tecles et al. (2007)
		0.66 (0.08)	5.70	Inter-assay	
		4.82 (0.08)	1.70	Intra-assay	
		4.85 (0.25)	5.10	Inter-assay	
		u.	3.31	Intra-assay	Hiss (2001)
		u.	10.27	Inter-assay	
TR-IFMA	Hp	0.36 (0.01)	4.81	Intra-assay	Gutiérrez et al. (2009)
		0.32 (0.01)	5.97	Inter-assay	

Hp = haptoglobin; SD = standard deviation; CV = coefficients of variation; u. = unknown.

traded, processed and consumed before it is tested on conspicuities. Hence, the goal of this study was to simplify and speed up the measurements of Hp and to lower the cost. A well suited approach to automate and simplify the analyses is the measurement by chip-based biosensors. Another Surface Plasmon Resonance (SPR)-based biosensor was already used to detect Hp in milk (Akerstedt, Björck, Persson Waller, & Sternesjö, 2006) and the flexible sam®5 system (Perpeet et al., 2006; Schlensog, Gronewold, Tewes, Famulok, & Quandt, 2004), measuring mass related phase shifts of Love waves, has already been used to detect proteins. Thus, it is suited to test the unpurified, Hp-containing meat juice, saliva, serum and also whole blood samples. In this article, the usability of this chip-technology to perform Hp measurements is tested. The results of Hp concentrations in the samples were checked by standard ELISA (Hiss, 2001). Advantages of such a biosensor approach are short detection times, low detection limits, high rates of automation and low costs for large numbers of samples (Cho & Park, 2006; Lan, Wang, Yin, Hoffmann, & Zheng, 2008).

The aim of this study was to develop and test a new measurement method for the quantification of Hp concentrations in meat juice samples of pigs.

2. Materials and methods

2.1. Collection and preparation of the samples

The meat juice was obtained by thawing frozen muscle samples. Within this study, muscle samples were taken from boars which have been investigated and watched during their growth as a first trial. All pigs used for these measurements had the same origin and genetic setup to minimize possible side effects. After electrical stunning, bleeding, scalding and splitting of the carcasses at a commercial slaughterhouse, the muscle samples were dissected from the diaphragmatic pillars and frozen in meat juice containers described by Christensen (2003) at −20 °C. By freezing the muscle samples, cell membranes were damaged and meat juice was obtained after thawing.

2.2. The biosensor system

The used biosensor was the chip-based sam®5 system (SAW instruments, Germany). This biosensor uses proprietary surface acoustic wave technology. A binding surface specific for porcine Hp has been developed on a standard gold chip. The biosensor measures phase shifts φ and amplitude alterations of longitudinal Love waves within a piezoelectric crystal. The analyte was bound to the surface by a specific ligand. The sam®5 allows to record directly the phase shift φ and an amplitude signal *A* of an applied longitudinal wave as a function of the quantity of bound molecules on the chip surface. A rigid mass load to the surface leads to a pure phase shift that is proportional to the mass density, whereas a fluid loading leads to a phase shift companied by an increased damping of the acoustic wave, amplitude attenuation (Perpeet et al., 2006). By this, it enables the analyst to separately interpret the mass loadings by the changes of φ and viscoelastic effect alterations by the changes of *A*. The measurements were performed at room temperature at a continuous buffer flow of 40 μL/min.

The results were compared to reference measurements by using a commercial Hp assay (RAIDASCREEN®Haptoglobin, R-Biopharm, Germany).

2.3. The ELISA reference of Hp concentrations

Hp contents were detected with a commercial ELISA, the RAIDASCREEN®Haptoglobin developed by Hiss (2001). It is based on the concept of a competitive ELISA with a secondary antibody, specific to the primary antibody. The CV of this assay is 5.7% at a concentration of 0.9 g/L, and the limit of detection is given with 0.033 g/L (product information).

2.4. Preparation of the chip surface

The measurement is based on the mass-related phase shift (φ) as a function of mobile Hp diluted in PBS running buffer, which binds to antibodies immobilized on the surface of a standard gold chip with a carboxymethyl dextran (CMD) surface. The chip contains five sensor elements (Perpeet et al., 2006). At each injection, phase shifts at defined time points are taken and compared. Antibodies were (1) directly immobilized via carbodiimide chemistry (Schlensog et al., 2004), or (2) indirectly bound via their Fc region to a recombinant protein A/G surface. Rabbit antibody solutions enriched with highly selective antibody against porcine Hp, or containing crudely mixed antibodies (reference) were applied to the sensor elements at 1:500 dilution. After preparation, the chip was placed into the sam®5 biosensor at 40 μL/min in phosphate buffered saline (PBS) pH 7.4. Hp samples were diluted in running buffer and injected into the buffer stream. Between injections, the antibody surface was regenerated to a baseline level using 10 mM Glycine pH 2.2 (surface 1), or with 10 mM Acetate buffer pH 4.5 (surface 2). The complete antibody layer was stripped off the protein A/G surface at pH 2.0, and fresh antibodies were applied for another set of experiments. By using an identical surface, the Hp responses could be compared directly. The antibody surface used was relatively stable for several days of continuous usage at conditions applied. This was tested by repeated injections at identical conditions.

2.5. Performed statistical analysis

To prove the accuracy of the new developed measurement method, coefficients of correlation (*R*) between concentrations measured by ELISA and detected phase shifts have been calculated by linear regression. All statistical analyses were performed by using the standard analytical software of the sam®5 system. Kinetic data were evaluated with the Origin 8.1 (Origin Lab, Northampton, MA, USA) based FitMaster. The FitMaster is a routine developed by SAW instruments to automate the kinetic analyses. Selected consecutive injections were cut out and integrated fits were applied. The resulting overlay plot and the individual fits following a 1:1 interaction of ligand and ligate are displayed in Fig. 2. The pseudo-first order kinetic constants (k_{obs}) as determined by the FitMaster were plotted versus calculated Hp concentrations. A linear best fit was applied using the equation shown with k_{on} = association rate constant (on-rate) and k_{off} = dissociation rate constant (off-rate). The average k_{off} [Unit in s^{-1}] equals the intersection with the y-axis. The slope of the fitted straight line is a measure of the k_{on} rate [Unit in $conc^{-1}\ s^{-1}$]. The dissociation constant (K_D) is calculated with $K_D = k_{off}/k_{on}$, as described by Gronewold, Baumgartner, Quandt, and Famulok (2006). Standard deviation (SD) and the according coefficients of variation (CV) were calculated for

the 13 samples based on three replicate measurements. CV and detection limit were compared with the reliable values ascertained by Tecles et al. (2007).

3. Results

3.1. Reference concentrations of Hp in samples measured by using ELISA

Hp samples from porcine meat juice were tested by ELISA as previously described. In Table 2 the Hp concentrations of 13 samples measured by ELISA and the coefficients of variation are given. For sample 1, the high CV of 18.5% indicates an over- or underestimation.

3.2. Immobilization of the antibody

Unpurified mixtures of rabbit serum were used in the ELISA experiments and were as well applied to the sensor chips, either from animals immunized with porcine Hp, or from control animals. The coating was performed externally. On recombinant protein A/G surfaces, the IgG fraction of the antibodies bound randomly based on their content in the serum, but directed via their Fc region. The antigen binding sites are presented to the mobile porcine meat juice contents. Antibodies coupled using carbodiimide chemistry were randomly bound to their exterior primary amines.

3.3. Proof of principles for the detection of Hp in meat juice by using biosensor technology

The linear regression of the measured phase shifts [°] and the Hp concentrations measured by ELISA for all tested meat juice samples are compared in Fig. 1. The presented phase shifts display the average of three measurements. SD was 0.17 and the CV was 5.5%.

In Fig. 1 Hp concentrations determined by ELISA were compared to the corresponding sam®5 signals. Lower concentrations measured by ELISA show a phase signal slightly above the linear regression and higher concentrations slightly below. However, the coefficient of correlation (R) is 0.98. This indicates the high quality of the measured phase signals, which correspond highly with the measured ELISA results as well as the linearity and accuracy of both methods.

In Fig. 2 an overlay plot of different concentrations caused by a serial dilution of one meat juice sample is shown. The concentrations have been calculated based on the Hp concentration of sample 11, the sample with the highest concentration. Sample 11 contains 214 mg Hp/L (see Table 2) measured by ELISA. Based on the molecular weight of 120,000 g/L, a Molar content of $1.43 * 10^{-9}$ M = 1.43 nM was calculated for the 1:1000 dilution of sample 11.

Table 2
Haptoglobin concentrations measured via ELISA.

Number	Hp [μg/mL]	CV [%]
1	39	18.5
2	71	10.0
3	75	7.2
4	82	2.3
5	99	0.6
6	103	3.2
7	122	7.6
8	131	3.3
9	151	3.9
10	163	1.2
11	167	1.6
12	199	6.3
13	213	7.5

Hp = haptoglobin; CV = coefficients of variation.

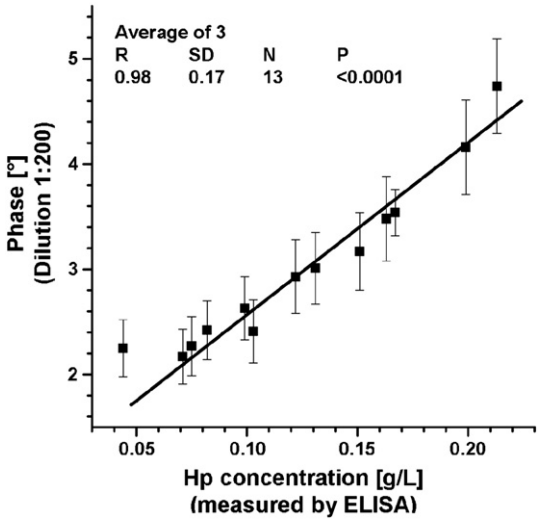


Fig. 1. Linear regression of ELISA results and phase shifts.

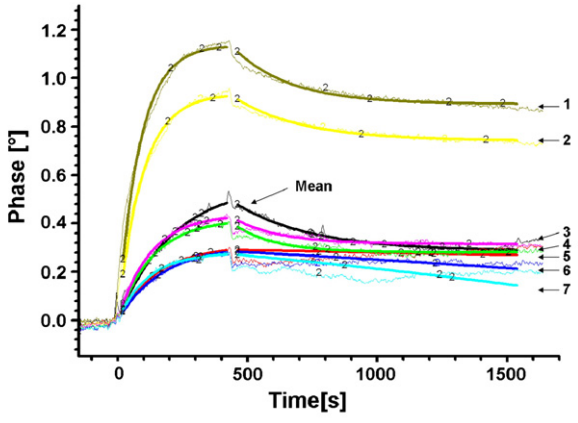
Accordingly, the Molar content for further dilutions 1:2000, 1:3000, 1:4000, 1:5000, 1:7500 and 1:10,000 was calculated (see Fig. 2).

For kinetic evaluation, the resulting curves were automatically exported into Origin 8.1 (Origin Lab), using the integrated FitMaster (SAW instruments), injections were cut and fits based on a 1:1 binding model were applied. The resulting overlay plot and the individual fits are displayed in Fig. 2.

In Fig. 3 the phase shifts display the dilutions of the sample. The coefficient of correlation (R) 0.989 was calculated by linear regression. Based on the pseudo-first order kinetic constants (k_{obs}) determined by the FitMaster and the calculated concentrations, a $K_D = k_{off}/k_{on} = 0.96$ nM was determined, assuming the concentrations of injected meat juice were calculated correctly.

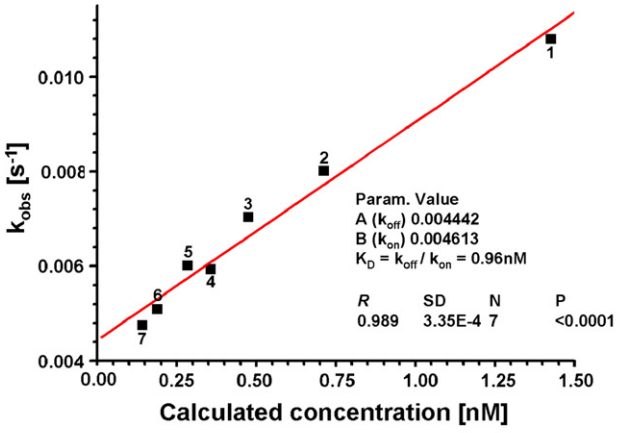
4. Discussion

In a first test, porcine Hp was measured from unpurified meat juice, which is a crude mixture of water, proteins, fat and other undefined contents. Thus, the measurement of one specific protein is a big



Sample	Dilution	Calculated Concentration [nM]
1	1:1000	1.43
2	1:2000	0.72
3	1:3000	0.47
4	1:4000	0.35
5	1:5000	0.29
6	1:7500	0.19
7	1:10000	0.14

Fig. 2. Overlay plot of phase shifts of sample 11 diluted to calculated Hp concentrations.



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3	1:3000	0.47
4	1:4000	0.35
5	1:5000	0.29
6	1:7500	0.19
7	1:10000	0.14

Fig. 3. Linear regression of sample 11 diluted to calculated Hp concentrations.

challenge. However, the used biosensor performed well and the coefficient of correlation ($R = 0.989$) is very high between the results of the two compared analytical methodologies. Further the SD and the CV are comparable with those measured by ELISA (Hiss, 2001; Tecles et al., 2007).

The sam®5 measurement results are linear, while the ELISA results show a slightly S-shaped curve response to applied concentrations (Hayashi et al., 2005). In a central interval, which is roughly linear, measurements are performed. At higher and lower concentrations, the results are leaving the quasi-linear regime. Lower concentrations show a phase signal slightly above the linear regression and higher concentrations slightly below. This can be attributed to the non-linearity of the ELISA method, introducing a small, but significant methodological error into almost all results (Hayashi et al., 2005). This sets the lower end of the limit of detection for the ELISA method to 20 mg/L. Contrarily – due to the linearity of the sam®5 signal – concentrations beyond the upper and lower limit of the ELISA method can easily be handled.

Due to the setup of the sam®5 system, with a few alterations on-site measurements are envisioned. A regeneration of the chip surface enables fast testing of multiple samples, which lowers costs as well as comparative measurements on identical surfaces. First tests to regenerate the surface by a simple pH shift and usage for several days showed very promising results. The chip containing five sensor elements, allows testing of four markers simultaneously, since the reference could be used on the remaining element for all markers. The calculated coefficients of correlation (R) and variability (CV) indicate that the sam®5 system has the same or improved potential to measure Hp levels in unpurified meat juice within a shorter analysis time than ELISA (Tecles et al., 2007). The nearly complete automated setup of the biosensor qualifies this method as a more robust method compared to ELISA and thus improves the comparability of results measured in different laboratories (Eckersall, 2002; Skinner, 2001).

5. Conclusions

Porcine Hp was successfully identified and quantified using the sam®5 system. The quasi-linear graph of the ELISA-vs-phase shift plot shows the possibility to standardize the system using reference samples. This would enable the calculation of the original concentration of unknown samples. The linear concentration range has to be defined. Since concentration of Hp is within a relatively small range for samples originating from blood, meat juice, or saliva, the necessary dilution can be standardized and even prepared in a sampling device destined for specific origins of samples.

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