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Research paper

Generation of an anti-NAGase single chain antibody and its application in a biosensor-based assay for the detection of NAGase in milk

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ABSTRACT

Bovine mastitis, an inflammation of the mammary gland in cows, is a major challenge for the dairy industry worldwide as it lowers milk yield, reduces milk quality and increases overall production costs. Early diagnosis is of the utmost importance. N-acetyl-β-D-glucosaminidase (NAGase) is an enzyme released into milk during inflammation and acts as an early indicator of mastitis. This paper describes the selection of anti-NAGase single chain fragment variable antibodies (scFv) from naïve human antibody libraries and their incorporation into an automated optical biosensor-based immunoassay to detect NAGase in milk. The scFv with the highest affinity for NAGase was first characterized by inhibition ELISA, followed by further evaluation using a surface plasmon resonance platform. Purified NAGase was immobilized on the surface of a CM5 chip and spiked NAGase milk samples were analyzed. The limit of detection for the assay for the assay was determined as 1 μg/ml.

1. Introduction

Bovine mastitis, an inflammation of the mammary gland in cows, is a major disease challenge for the dairy industry worldwide. Mastitis affects the quality of milk and causes a loss in milk yields, resulting in increased milk production costs. It also presents health risks to other cows due to the possibility of infections, resulting in high costs associated with treatment or animal culling in chronic cases. Clinical mastitis is easy to detect due to the appearance of visible signs associated with the disease. Subclinical mastitis, however, is difficult to detect due to the absence of any visible indications and requires the availability of a rapid screening test for early-onset disease detection (Viguier et al., 2009).

Current diagnostic methods involve the estimation of somatic cell count (SCC) as an indicator of inflammation, in milk, using large equipment such as a Fossomatic cell counter or on-site tests such as the California Mastitis Test (CMT). The analysis of biomarkers associated with the onset of disease provides a sensitive method for diagnosing the different stages of mastitis. Enzymes such as NAGase and lactate dehydrogenase (LDH) are known to increase during inflammation and act as early indicators of mastitis. Colorimetric and fluorometric assays have been developed to measure the elevated concentration levels of these enzymes in milk (Larsen, 2005; Kitchen et al., 1978). Immunoassays were used for the detection of the acute phase proteins (APP) haptoglobin (Hp) and serum Amyloid A (SAA), which increase significantly in milk during inflammation (Gronlund et al., 2003). An enzyme linked immunosaorbant assay (ELISA) has been developed for Hp with a detection limit of 0.07 μg/ml in milk and serum (Hiss et al., 2004) and a commercially available solid phase sandwich ELISA has also been developed against SAA (Szczubial et al., 2008).

Many of these assays are time consuming when used for large scale analysis. Biosensors offer an alternative approach to

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these diagnostic methods. Biacore systems use the optical phenomenon of surface plasmon resonance (SPR) to characterize the label free interactions of proteins with other proteins, peptides, nucleic acids, lipids and small molecules in real time. The detection principle of SPR used in these systems is an electron charge density wave phenomenon that arises at the surface of a metallic film when light is reflected at the film under certain conditions. Resonance occurs as a result of momentum that is transformed from incident photons into surface plasmons and is sensitive to the refractive index of the medium on the opposite side of the film from the reflected light. When Biacore is used to measure protein interactions, one of the interactants (ligand) is immobilized onto the surface of a sensor chip consisting of a thin metal film applied to a glass surface and the other interactant (analyte) is passed over the surface in solution through an integrated micro-fluidic system. Binding of the analyte to the ligand causes a change in angle of the reflected light at the surface of the chip that is measured and this measurement is proportional to the molecular mass on the chip surface (Murphy et al., 2006). Compared to other diagnostic detection systems, Biacore has the advantage of being automated and rapid, each sample requiring around 5–10 min to analyse. Several groups have previously used Biacore to analyze different components in milk such as HP (Akerstedt et al., 2006), progesterone (Gillis et al., 2002), and beta-lactams (Gustavsson et al., 2004).

The production of antibodies is an integral part of the development of any immunoassay, and in this study, the phage display technology was used to isolate scFv antibody fragments against NAGase from the human Tomlinson scFv library. The Tomlinson library consists of more than 100 million different scFv fragments cloned into a phagemid vector. The use of recombinant antibody libraries combined with high throughput screening techniques offers an alternative to the traditional immunization of animals and hybridoma technology to specifically isolate monoclonal antibodies against diverse targets. The scFv isolated was successfully incorporated into an automated optical biosensor-based immunoassay for the detection of NAGase.

2. Materials and methods

2.1. Reagents

Analysis was carried out on an SPR instrument (Biacore 3000™, GE Healthcare, Uppsala, Sweden) using a CM5 sensor chip. The running buffer for all Biacore experiments was the HBS buffer, pH 7.4, containing 10-mM HEPES, 150-mM NaCl, 3.4-mM EDTA, and 0.05% (v/v) Tween 20. The HBS buffer was filtered (pore size of 0.22 μm) and degassed using a Millipore filtration apparatus (sintered glass filtration unit) immediately before use. The Human Single Fold scFv libraries I + J (Tomlinson I + J), *Escherichia coli* TG1 and HB2151 were provided by the MRC Gene Services. All other reagents used were of analytical grade and were purchased from Sigma Aldrich Ltd., Dublin, Ireland.

2.2. NAGase purification

All manipulations were carried out at 4 °C. After brief washing in a homogenization buffer (20 mM Tris/HC1, pH 7.4), a bovine spleen (100 g) was skinned, cut into small pieces and suspended in 100 mls of homogenization buffer containing 1 ml of 10× protease inhibitor cocktail. The spleen was homogenized in a Waring blendor (2 min at full speed). The homogenates were then centrifuged at 18500×*g* for 20 min. The supernatant was collected and filtered through a Whatmann filter paper 1 and, subsequently, through a 0.45-μm filter. The filtered supernatant was purified using FPLC (ÄKTA™ Explorer 100 with a UV-900 monitor) by anion exchange chromatography, followed by gel filtration. Anion exchange chromatography was performed on a Resource Q 1-ml column using 20-mM Tris-HCl, pH 7.8, (buffer A) and 20-mM Tris-HCl, pH 7.8, containing 0.5-M NaCl (buffer B). Samples were eluted from the column, at a flow rate of 1 ml/min, using step gradients of 0%–20%, 20%–30%, 30%–40%, 40%–50% and 50%–100% (v/v) of buffer B and 3-ml fractions were collected. The NAGase enzymatic activity assay was performed on all fractions, showing an absorbance at 280 nm. Fractions showing NAGase activity were pooled and applied to a HiLoad™ Superdex 200 prep grade column and gel filtration chromatography was performed using phosphate buffered saline (PBS) at a flow rate of 0.5 ml/min for a duration of 700 min. Fractions of 5 ml were collected and again the NAGase enzymatic activity assay was performed on all fractions, showing an absorbance at 280 nm.

2.3. Antibody selection

The panning procedure was performed on immunotubes coated with purified NAGase protein (50 μg/ml in round1, 25 μg/ml in round2, 10 μg/ml in round 3 and 1 μg/ml in round 4). The immunotubes were blocked with 2% MPBS [2% (w/v) marvel milk powder in PBS] for 2 h at room temperature. The immunotubes were then washed three times with PBS and 10^{13} Tomlinson phage library diluted in 4-ml MPBS was added. After 2 h of incubation at room temperature, non-binding phage was washed away with 0.1% (v/v) PBST [PBS containing 0.1% (v/v) Tween-20] 10 times. The remaining phage was eluted with 500 μl of trypsin-PBS. TG1 cells (1.75 ml) at an OD_{600} of 0.4 nm were infected with 250 μl of eluted phage for 30 min at 37 °C without shaking. Four fold serial dilutions of the phage infected TG1 cells were made, starting with a 1 in 10 dilution, and 10 μl of each dilution was spotted onto TYE plates containing 100 μg/ml ampicillin and 1% (w/v) glucose. Plates were incubated at 37 °C overnight, and the next day, the phage titer was determined by counting the clones. The remaining phage infected TG1 cells were spread onto TYE plates and incubated at 37 °C overnight. The next day, bacteria on the plates were scraped and added to 100 ml of 2× TY containing 100 μg/ml of ampicillin and 1% (w/v) glucose for amplification. The amplified phage was rescued by the helper phage M13K07 for the next round of selections. Four rounds of selections were performed in total.

2.4. Polyclonal phage ELISA

Nunc ELISA plates (Nunc A/S, Roskilde, Denmark) were coated with 100 μl of 5-μg/ml NAGase in PBS for 1 h at 37 °C. The plate was washed three times with PBS and then blocked with 2% MPBS for 2 h at room temperature. PEG precipitated phage [10 μl of phage and 90 μl of 2% (w/v) MPBS] from each

round of selection was added to the plate and the plate was then incubated for 1 h at room temperature. The plate was washed with 0.1% (w/v) PBST and 100 μl of a 1 in 3000 dilution of HRP anti-M13 (Sigma Aldrich) in 2% MPBS was added. The plate was incubated for 1 h at room temperature and then was washed three times with 0.1% (w/v) PBST and three times with PBS. The assay was developed using tetramethyl benzidine (TMB) for 20 min and stopped with 10% (v/v) HCl solution, and the absorbance was read at 450 nm.

2.5. Production of soluble antibody fragments

From the fourth round of selections, 10 μl of eluted phage was used to infect 200 μl of exponentially growing HB2151 bacteria ($OD_{600} = 0.4$) for 30 min at 37 °C. From this step, four fold serial dilutions were prepared and 50 μl of each dilution was plated out onto TYE plates containing 100-μg/ml ampicillin and 1% (w/v) glucose. The plates were incubated overnight at 37 °C, and the following day, individual clones were picked and used to inoculate 100 μl 2× TY containing 100-μg/ml ampicillin and 1% (w/v) glucose in a 96-well plate. The plate was incubated overnight at 37 °C with shaking (250 rpm). The following day 2 μl from this plate was transferred to a fresh 96-well plate containing 200 μl 2× TY containing 100-μg/ml ampicillin and 0.1% (w/v) glucose. The cells were grown with shaking (250 rpm) at 37 °C until an OD_{600} of approximately 0.9 had been reached. Twenty-five microliters of 2× TY containing 100-μg/ml ampicillin and 9-mM IPTG (final concentration 1 mM IPTG) was added to the plate. The plate was incubated overnight with shaking (250 rpm) at 30 °C. The following day the plate was spun at 3000 g for 10 min and 50 μl of supernatant containing soluble scFv fragments were used in ELISA as previously described, with the exception of the use of 3% (w/v) BSA–PBS to block and the use of a 1 in 3000 dilution of Protein A HRP (Pharmacia) to detect soluble monoclonal scFv fragments.

2.6. Antibody purification and determination of species composition by size-exclusion HPLC

Anti-NAGase scFv clones were grown in 500 ml of Terrific Broth (TB) containing 100-μg/ml carbenicillin at 37 °C at 200 rpm until an OD_{600} of 0.5–0.6 was reached. At this stage 500 μl of 1-M IPTG was added to give a final concentration of 1-mM IPTG and the culture was incubated at 30 °C overnight at 200 rpm. The culture was then spun at 3000 g for 10 min and the pellet was resuspended in 5 mls of lysis buffer (50-mM NaH_2PO_4, 300-mM NaCl, 10-mM imidazole pH 7.5) and put on ice. The sample was kept on ice and sonicated at an amplitude of 40% for 6-s pulses for 3 min. The cell extract was centrifuged at 18500×*g* for 20 min, the supernatant was filtered through a 20-micron filter and the scFv was purified using immobilized metal affinity chromatography (IMAC). IMAC was performed using a 1-ml Ni-NTA Agarose (Qiagen GmbH) using 4 column volumes (CV) of running buffer (50-mM NaH_2PO_4, 300-mM NaCl, and 10-mM imidazole pH 7.5), 8 CV of wash buffer A (50-mM NaH_2PO_4, 1-M NaCl, 10% glycerol, 20-mM imidazole, and 1% triton X-100 pH 7.5) and 16 CV of wash buffer B (50-mM NaH_2PO_4, 300-mM NaCl, and 20-mM imidazole pH 7.5). The protein was eluted with 5-ml elution buffer (50-mM NaH_2PO_4, 300-mM NaCl, and 250-mM imidazole pH 7.5) and fractions were collected in 0.5-ml fractions in sterile eppendorf tubes. Protein concentrations were measured at 280 nm and the fractions containing protein were pooled and buffer exchanged for 1× PBS using Zebra™ desalt spin columns (Pierce, Rockford, IL).

High-performance liquid chromatography (HPLC) assays were performed on a Shimadzu Prominence LC system (Mason Technology), and all water selected for use was of HPLC grade (18.2 mΩ) and purified using a Millipore purification system. The species composition of solubly expressed and IMAC-purified antibody fragments (e.g., monomeric or dimeric) was determined by size-exclusion chromatography. Separations were performed on a Bio-Sep-SEC-S2000 stationary phase (Phenomenex; 300×7.8 mm), with 1× PBS selected as a mobile phase. All samples (50 μl) were assayed at a flow rate of 0.5 ml/min using UV detection (280 nm). The conformation of individual samples was determined by referencing against a range of protein standards (Agilent), i.e., bovine thyroglobulin (670 kDa), immunoglobulin G (IgG; 150 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa). All analyses were performed in triplicate, with samples interspersed with water (HPLC grade).

2.7. Inhibition ELISA

Nunc ELISA plates were coated with 100-μl NAGase (Sigma Aldrich) at a concentration of 2 μg/ml in PBS and incubated for 1 h at 37 °C. Plates were washed three times with PBS and then blocked with 200 μl PBS containing 3% (w/v) BSA for 1 h at 37 °C. While the plates were blocking, an inhibition reaction was set up. For this step, NAGase (Sigma Aldrich) was diluted in PBS at concentrations of 0.625 μg/ml, 1.25 μg/ml, 2.5 μg/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml and 40 μg/ml. Fifty microliters of each dilution was incubated with 50 μl of lysate from each scFv clone. Plates were washed once with PBS, 100 μl of the inhibition reaction was added and the plate was incubated at 37 °C for 1 h. The plates were washed three times with 0.05% (v/v) PBST and three times with PBS, and 100 μl of a 1 in 3000 dilution of Protein A HRP was added to the plates for 1 h at 37 °C. Plates were washed three times with 0.05% (w/v) PBST and three times with PBS. The assay was developed using TMB for 20 min and stopped with 10% (w/v) HCl solution, and the absorbance was read at 450 nm.

2.8. Immobilization of NAGase on the CM5 sensor chip surface

The carboxymethylated dextran surface was activated by injecting equal volumes of 100-mM NHS (N-hydroxysuccinimide) and 400-mM EDC [N-ethyl-(dimethyl-aminopropyl) carbodiimide hydrochloride] at a flow rate of 10 μl/min for 10 min across the chip surface. NAGase was diluted in 10-mM sodium acetate, pH 4.6, to a final concentration of 50 μg/ml and passed over the activated chip surface at a flow rate of 5 μl/min. Any unreacted NHS groups were capped and noncovalently bound proteins were removed by the injection of 1-M ethanolamine hydrochloride, pH 8.5, for 7 min. The surface was regenerated three times with 20-mM NaOH before use.

2.9. SPR inhibition assay

For the inhibition assay, NAGase (Sigma Aldrich) was spiked into milk at concentrations of 0.62 μg/ml, 1.25 μg/ml,

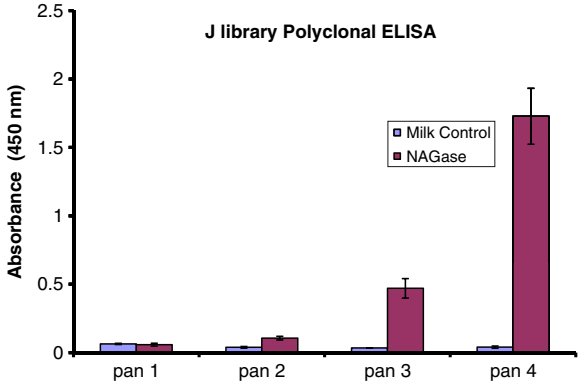

Fig. 1. Binding of polyclonal phage scFvs to NAGase and milk marvel from four rounds of selections. All assays were performed in triplicate. Data points represent the mean ± standard deviation.

2.5 μg/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml and 80 μg/ml and was incubated with an equal volume of anti-NAGase scFv lysate prior to injection over the surface of the CM5 sensor chip immobilized with NAGase. A custom wizard method was written, where each sample was injected randomly with respect to concentration over the CM5 chip at an optimized flow rate of 10 μl/min over a period of 6 min. Report points were recorded before and after sample injection, and the surface was regenerated with two 30-s pulses of 10-mM NaOH at a flow rate of 10 μl/min. The binding response for each reaction was recorded and a calibration curve was constructed. The limit of detection of was defined as three times the standard deviation of the blank. For intraday studies, replicates (n = 5) were prepared and assayed on the same day. For interday studies three sets of replicates were prepared and assayed on separate days under identical assay conditions.

3. Results

NAGase protein was used to direct the diversity of the human single fold scFv libraries I + J (Tomlinson I + J) for the selection of human scFv anti-NAGase- specific antibodies. Four rounds of selections were carried out, with decreasing concentrations of NAGase in each round. Populations of phage produced at each round of selection were screened for binding to NAGase to identify polyclonal phage antibodies (Fig. 1).

Phage from round 4 of selections was used to infect HB2151 cells and soluble antibody expression was induced with IPTG. Expressed soluble scFv clones were then screened for binding to NAGase and BSA. From 96 clones screened, 45 clones produced soluble antibody fragments that gave positive binding to NAGase compared to the BSA control (Fig. 2).

The 45 positive soluble clones identified were next tested for their inhibitory activity by ELISA. For this step, NAGase was diluted in PBS at concentrations of 0.625 μg/ml, 2.5 μg/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml and 40 μg/ml. Fifty microliters of each dilution was incubated with 50 μl of lysate from each scFv clone at the appropriate dilution, as determined by their titer (data not shown). The reactions were added to an ELISA plate coated with NAGase. The observed results were used to generate a calibration curve for each of the clones identified. The best scFv identified designated J12 is shown in Fig. 3. To further characterize the scFv clone J12, the species composition was subsequently determined by size exclusion HPLC. Dimeric and monomeric species were readily detected, with retention times of 15.7 and 17.9 min, respectively. The ratio of monomeric to dimeric composition was estimated based on the calculation of peak areas, with the recombinant antibody deemed to be predominantly monomeric based on this analysis (Fig. 4). This scFv was taken forward for further SPR-based assay development.

An SPR-based assay was developed for the detection of NAGase in pasteurized milk. Approximately 7629 response units (RU) of NAGase protein was successfully immobilized onto the surface of a CM5 sensor chip. Regeneration scouting studies revealed that 10 mM NaOH efficiently removed bound antibody while retaining immobilized NAGase activity. By incorporating a factorial approach to assay design, multiple antibody concentrations, flow rates and contact times were

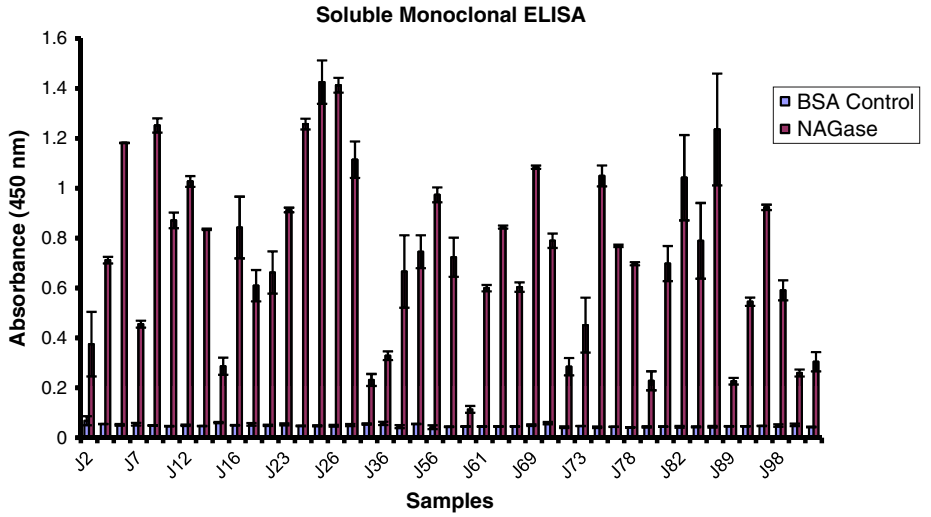

Fig. 2. Binding of soluble monoclonal scFvs to NAGase and BSA. All assays were performed in triplicate. Data points represent the mean ± standard deviation.

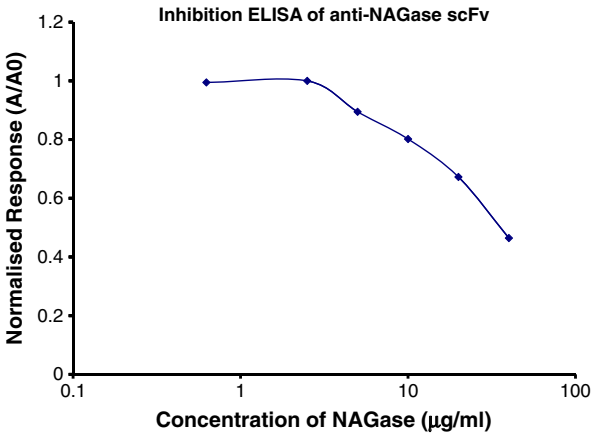

Fig. 3. scFv clone J12 shows inhibitory activity against NAGase.

evaluated (results not shown). For this assay a 1 in 4000 dilution of purified anti-NAGase scFv at a flow rate of 10 µl/min for 6 min yielded optimum results.

An inhibition assay for detecting NAGase in spiked milk was developed using the SPR platform, Biacore 3000. NAGase was spiked into milk at concentrations of 0.625 µg/ml, 1.25 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml and 80 µg/ml. Equal volumes of each dilution was incubated with an equal volume of purified anti-NAGase scFv. Each sample was injected randomly with respect to the concentration over the surface of the surface of the CM5 sensor chip immobilized with the NAGase protein. The binding response for each reaction was recorded and a calibration curve was constructed by plotting the change in response to each reaction against the known concentrations of NAGase spiked in milk for each reaction. The linear range for this assay was 2.5–40 µg/ml (25–400 U/L; Fig. 5). The limit of detection was defined as three times the standard deviation of the blank and was determined to be 1 µg/ml (10 U/L) for the assay.

To determine the accuracy of the assay, intraday and interday assay variability studies were performed. Three sets of standards were prepared and incubated with antibody before being assayed. The percentage CVs for the intraday and interday assays were determined and ranged from 3.63% to 11.88% for the intraday assay and from 3.13% to 11.17% for the

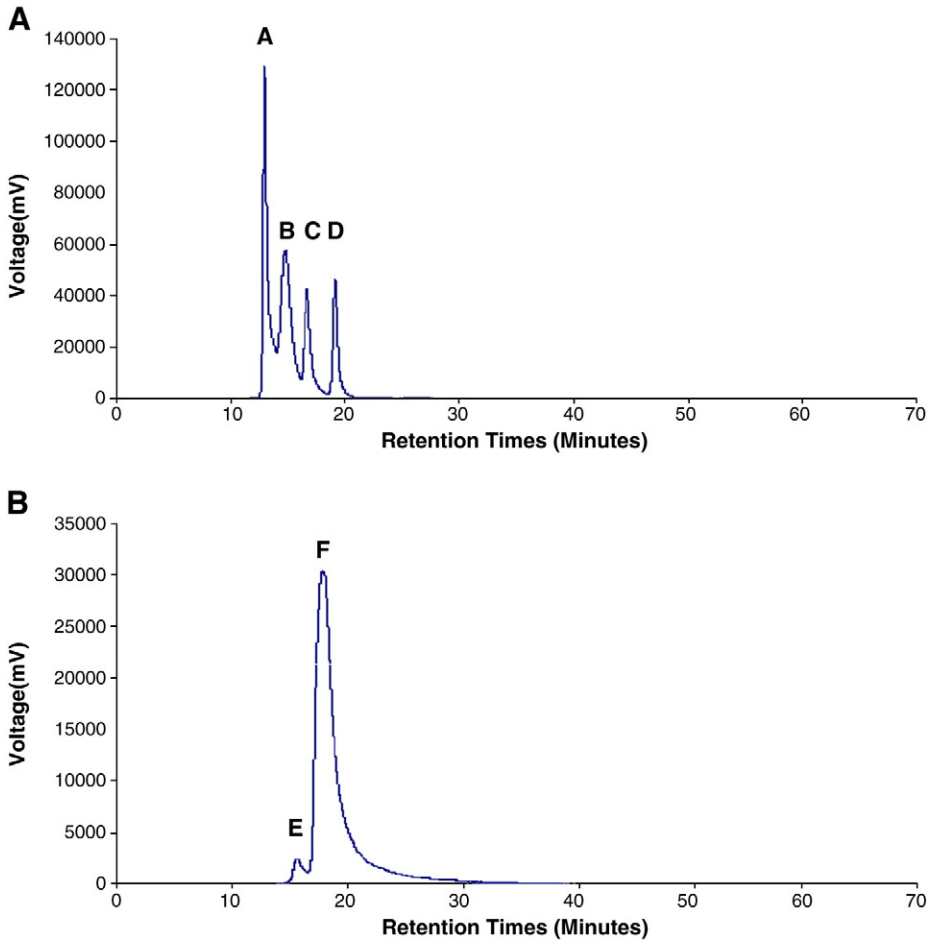

Fig. 4. Species composition of the anti-NAGase scFv clone J12. (A) Protein standards were selected for size determination, and retention times were given as follows: bovine thyroglobulin (A; 12.9 min), immunoglobulin G (B; 14.8 min), ovalbumin (C; 16.7 min) and myoglobin (D; 19.2 min). (B) The scFv comprised a mixture of dimeric (E) and monomeric (F) species in a ratio of 3.27:96.73%, the latter with an estimated molecular weight of 28 kDa.

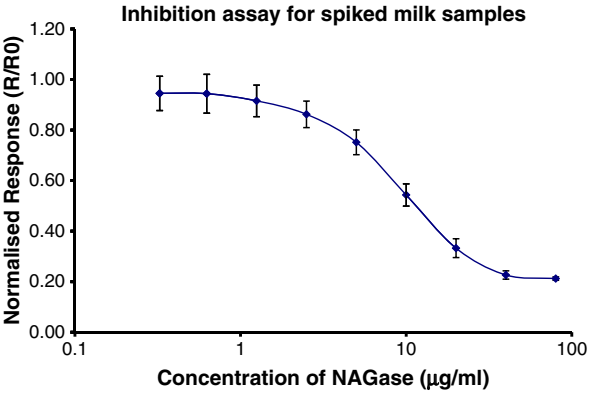

Fig. 5. Standard curve of NAGase in milk, produced on Biacore 3000. The assays were performed in triplicate and the error bars indicate the standard deviations.

interday assay, indicating that the assay had a good degree of precision.

4. Discussion

This paper describes the generation and development of anti-NAGase scFv antibody fragments and their incorporation into an SPR-based immunoassay for the detection of NAGase in milk. A key requirement for the development of this assay was the production of a recombinant antibody using phage display technology. The scFv isolated in this study contained mainly the monomeric form of scFv and this is the first time that a recombinant antibody against bovine NAGase has been reported.

The anti-NAGase scFv was successfully incorporated into SPR-based immunoassay, which could reliably detect NAGase in the complex matrix, milk. NAGase spiked into pasteurized milk samples blocked the binding of recombinant anti-NAGase scFv antibody fragments to immobilized NAGase. Thus, there is an inverse relationship between the concentration of the NAGase in the spiked sample and the magnitude of the response recorded on the Biacore. The assay system could easily quantify samples at concentrations greater than 1 µg/ml (Fig. 6). To verify that the assay was fit for purpose, a calibration curve was generated for the measurement of NAGase concentration and the reproducibility of the assay monitored by repeated testing. Intraday and interday assay CVs were lower than 12%. The SPR-based biosensor described in this assay had a similar limit of detection (10 U/L) in milk as a biosensor using a screen-printed carbon electrode to detect NAGase in PBS through its ability to convert 1-naphthyl N-acetyl-β-D-glucosaminidine to 1-naphthol (Pemberton et al., 2001). The screen-printed carbon electrode assay gave a linear response in the range 3.1–108 U/L NAGase, in comparison to the linear response of 2.5–400 U/L observed in this assay.

SPR sensors offer several advantages over enzymatic assays commonly used for the detection of NAGase. They require very low amounts of reagents, use unlabelled antibodies, and are reusable. A Biacore assay for the detection of the mastitic biomarker haptoglobin (Hp) was developed by Akerstedt et al. (2006). This competitive assay used SPR to

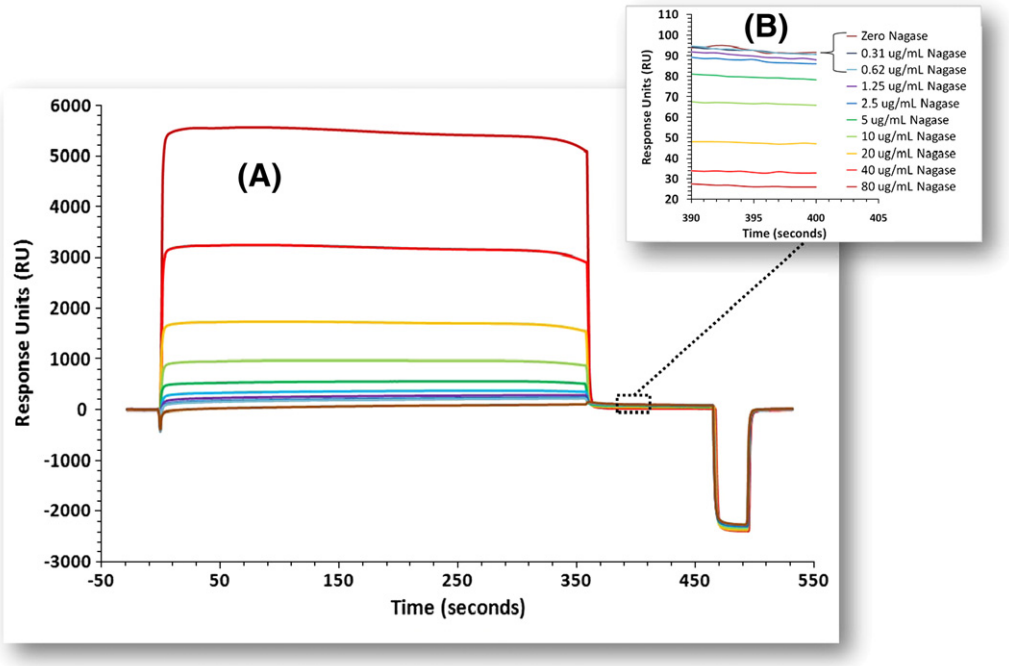

Fig. 6. Overlay plot demonstrating the decrease in binding of affinity-purified anti-NAGase scFv antibody to the immobilized NAGase on the chip with increasing free NAGase concentrations. The plot shows the referenced raw data of 10 binding cycles overlaid and normalized with respect to the injection time. Even though large refractive index increases were observed due to high NAGase concentrations (observed in graph A), a characteristic decrease in binding level was observed after incubation in buffer (graph B). Graph B shows the response level decrease for scFv antibody mixed with varying concentrations of NAGase.

monitor the interaction between Hp, which was immobilized onto the chip surface, and hemoglobin (Hb) to discriminate between subclinical mastitic and nonmastitic milk. Haptoglobin binds strongly to Hb; therefore, by mixing the milk sample with Hb, any Hp present in the milk sample binds to the Hb, thus preventing binding of the Hb to the immobilized Hp. In the absence of Hp (i.e., in an uninfected milk sample), Hb binds to the immobilized Hp, resulting in a positive signal, which is reduced in correlation to the levels of Hp present in the milk. However, most milk samples contain some blood (and, therefore, Hb), particularly in clinical mastitis, and the Hp can bind to the Hb in the milk and result in an inaccurate assessment of the concentration of Hp in the milk sample.

Ultimately, the aim is to develop this assay for use in the detection of mastitis in milk samples from infected cows. The levels of NAGase are 25 U/L in healthy milk and increase to 30 U/L in mastitic milk (Piccinini et al., 2007; Tamburini et al., 2009), and although these values are well above the limit of detection of the described assay (10 U/L), the ability of this assay to discriminate between mastitic and healthy samples will require further investigation.

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