Novel electrochemical identification and semi quantification of bovine constituents in feedstuffs

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Abstract

Identifying contaminating bovine constituents in feed has been a major means to help prevent the spread of bovine spongiform encephalopathy (BSE). The phenomenon of DNA aggregation induced by Hoechst 33258 in conjunction with the change in anodic current measurement was, for the first time, applied for bovine DNA detection in feedstuffs. By using the PCR amplification system specific to bovine parathyroid and common 12S rRNA genes, anodic current peaks measurements of these PCR products on linear sweep voltammetry could be determined. Anodic peaks measurement of bovine parathyroid gene among ruminant meat and pet foods containing bovine constituents were at 1.18–1.52 mA while anodic peaks among non bovine samples were greater than 1.78 mA. In the study, anodic current peaks greater than 1.75 mA could be used to distinguish non-bovine from other samples in a qualitative analysis. For quantitative analysis, bovine content was measured using the comparative ratio between copy number of bovine parathyroid and 12S rRNA genes. This ratio reflected the proportion of target bovine cells to total cell numbers. In the experiment, contents of bovine constituents in four kinds of tested pet foods were 10.88, 8.76, 6.39 and 2.69%. Compared with the first two samples on which defined content had been addressed, the estimated content with 90.66 and 87.60% accuracy could be obtained, respectively.

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1. Introduction

Enforcing the regulation on meat blood and bone meal in feed for farm animal production especially ruminants is considered an important measure to prevent unsafe foods due to the risk of bovine spongiform encephalopathy [1].

For this specific purpose, a microscopic examination of animal constituents in animal feeds is the only official method available [2]. However, this method requires skillful and experienced staff and cannot identify the animal species involved. The alternative application of immunological methods was similarly less advantageous since the identification of heat-treated components contained in feedstuffs was practically difficult due to thermal protein denaturation [3].

DNA based methods have been demonstrated to be a reliable tool for food and feed analysis [4] with advantages such as a degree of specificity and applicability even in thermally processed products.

So far, several DNA methods based on PCR in combination with restriction enzymes (PCR–RFLP) or PCR alone (PCR/RAPD) have been developed [5,6]. Result analysis of those methods, however, requires further steps of analysis on restriction endonuclease or sequence or banding profiles, which are time consuming and provide only qualitative data.

Recently, a biosensor detection system using electrochemistry has been demonstrated for nucleic acid based diagnosis [7]. For detection, the system needs a biomolecule that has a highly specific recognition element and a transducer that converts a molecular recognition event into a quantifiable signal. A demonstration of signal transduction using electrochemicals had been carried out using a DNA probe, a modified gold electrode, and Hoechst 33258 as a label [8]. However, all of those methods required laborious steps of immobilizing the...
probe DNA on the surface of the electrode before hybridization.

In a previous report, our group had developed a method based on electrochemical biosensor that required no probe and immobilization steps [9]. The method employed the aggregation phenomenon of the target DNA in the presence of Hoechst 33258. The aggregation of the DNA provides a clue to quantifying DNA at a trace level through the measurement of anodic current peaks. Anodic current decreases in proportion to the titration of dsDNA aggregations. Success in the fabrication process of the electrode unit, which can be integrated into the system for detection of DNA in reaction solution without DNA purification, enables the detection of target DNA in a rapid, less intensive, and cost effective fashion.

In this report, we applied DNA aggregation induction by Hoechst 33258 to the bovine DNA detection in feedstuffs by integrating the capability and specificity of the PCR technique for a qualitative analysis. We then measured the amplification ratio between a known single DNA copy of bovine parathyroid gene (Pth) and the common eukaryotic 12S ribosomal RNA (rRNA) gene to find a relationship between both. The content of bovine constituents was calculated based on number of bovine related cells detected via Pth gene per total cells detected via 12S rRNA gene. This is the first DNA detection system that demonstrates the application of DNA binding ability of Hoechst 33258 molecules to target DNA materials, and the application of the electrochemical biosensor principle in the detection of bovine DNA species in feedstuffs.

2. Experimental procedures

Meats, feedstuffs and DNAs (Table 1) were extracted from samples obtained from commercial sources using 300 mg samples as starting materials. Confirmation of the meat species was according to Wolf et al. [10]. Design of primers was based on the bovine Pth gene (K01938) and a 12S rRNA gene [8,21]. Selected primers, (5′-tataaaagtcacattgaagggtctacag-3′ and 5′-tgtaagaaagaactcatggaaacttaaa-3′ for the Pth gene and

Table 1
Detail descriptions of samples tested, and the comparative results between qualitative detection of bovine specific Pth DNA through anodic current peaks measurement and results from gel electrophoresis

| Sample no. | Components in label or in notification (major to minor) | Characteristic | Country of origin | Confirmation of meat species | Anodic peak (µA), (SD), and judgement | DNA detected | Result bovine or non-detected |
|------------|---------------------------------------------------------|----------------|------------------|----------------------------|----------------------------------------|--------------|-------------------------------|
| 1. Cloned target DNA | – | DNA solution | – | ND | 0.88(0.09)/+ | + | Bovine positive control |
| 2. Soybean genome DNA | – | DNA solution | – | ND | 2.06(0.08)/- | – | Non-detected negative control |
| 3. Non-template control | – | – | – | ND | 2.02(0.07)/- | – | Non-detected control |
| 4. Poultry meat | – | Sliced meat | Japan | Poultry | 1.83(0.06)/- | – | Non-detected |
| 5. Ruminant meat | – | Sliced meat | Japan | Bovine | 1.18(0.06)/+ | + | Bovine |
| 6. Pork meat | Porcine | Sliced meat | Japan | Porcine | 1.82(0.06)/- | – | Non-detected |
| 7. Pork meal | Porcine | Powder | Netherlands | Porcine | 1.80(0.08)/- | – | Non-detected |
| 8. Porcine protein | Porcine | Powder | Denmark | Porcine | 1.76(0.05)/- | – | Non-detected |
| 9. Porcine meal | Porcine | Powder | Denmark | Porcine | 1.78(0.07)/- | – | Non-detected |
| 10. Poultry by-product meal | Poultry | Pellet | Netherlands | Poultry | 1.81(0.08)/- | – | Non-detected |
| 11. Nutrition | Poultry | Pellet | France | Poultry | 1.85(0.05)/- | – | Non-detected |
| 12. Pet food pellet A 15% protein | Bovine | Pellet | Australia | Bovine and poultry | 1.36(0.06)/+ | + | Bovine |
| 13. Pet food pellet B 15% protein | Bovine poultry | Pellet | Australia | Bovine and poultry | 1.36(0.06)/+ | + | Bovine |
| 14. Pet food soup 8% protein | Bovine Poultry | Cube meat | Netherlands | Bovine and poultry | 1.42(0.08)/+ | + | Bovine |
| 15. Pet food pellet 17.6% protein | Bovine poultry | Pellet | Japan | Poultry and bovine | 1.52(0.06)/+ | + | Bovine |
| 16. Pet food slice and pellet 30% protein | Mixed species | Pellet | Australia | Poultry and porcine | 1.88(0.06)/- | – | Non-detected |

ND, not determined; +, detected; –, not detected.

a Tested according to Wolf et al. [10].

b Determined on agarose gel electrophoresis.
5'-gcacg(t/c)aca(t/c)accgcccgtcaccctc-3' and 5'-tcc(a/g)gta(t/c) (a/g)cttacctgtttacctg-3' for the 12SrRNA gene [11], were based on criteria of specific DNA amplification to Pth gene and 12S rRNA gene without leaving non-specific products. Several PCR conditions including the annealing temperature and the Mg$^{2+}$ ion concentration were also adjusted to guarantee amplification of specific DNAs.

For identification, the DNA was first extracted as described in the DNA Blood Kit (Qiagen, USA) manufacturer’s protocol without further purification and confirmed for its ability to be amplified by PCR. For the qualitative test, positive control DNA, negative control DNA, and target samples, were then amplified in 50 µL reactions using 1 µL of DNA, 2 U of KOD Plus (Toyobo, Japan) with 50 µM MgCl$_2$ for Pth gene and 25 µM MgCl$_2$ for 12S rRNA gene, 10 pmol each of primers. Amplification was carried out under the PCR conditions of pre-incubation at 94 °C 5 min, 35 cycles of 94 °C 30 s, 68 °C 1 min and post-incubation at 68 °C for 10 min. Detection of electrochemical signals induced after mixing 20 mM Hoechst 33258 with PCR products was further carried out in PBS buffer using linear sweep voltammetry (LSV) and a fabricated sensor based on the screen printed carbon (area:1.96 mm$^2$) and Ag/AgCl as the reference electrode. All measurements were performed in quadruplicates at 37 °C.

For the DNA quantification, cloned portions of bovine Pth and the 12S rRNA genes in pCR4™ (Invitrogen, USA) were used as the known copies of reference, and only bovine positive samples from the qualitative study were further tested. The DNA was further purified using Micro Spin S200-Column (Amersham, USA). Measurements were taken after 30 cycles of amplification of the reference Pth and 12S RNA genes, with conditions as described earlier, at 100, 10, 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001 ng of DNA templates compared with those of the positive samples from the qualitative test. Calibrations were based on relationships between copy numbers of the Pth gene and the 12S rRNA gene to each anodic current peak drawn against copy numbers via standard curve method [12]. Content of bovine constituents was determined based on the ratio number of bovine related cells per total cell numbers estimated from copy numbers of the Pth gene and the 12S rRNA gene, respectively.

3. Detection system using the Pth and the 12S rRNA genes

The PCR system based on the amplification of specific Pth gene and 12SrRNA gene had been established. When the Pth gene and the 12SrRNA gene were amplified with 50 and 25 µM MgCl$_2$, respectively, expected DNA fragments of 165 and 115 nt, without non-specific products, could be obtained. The system left no trace of non-specific products, even when DNAs from poultry and porcine species, common sources of raw materials for feedstuffs production, were used as templates (Fig. 1). Sensitivity, when observed through the diluted DNA, revealed a detectable range of DNA at the amount as low as 0.01 ng in samples (Fig. 2). Reproducibility via specificity of PCR products at more than 20 attempts of amplification was also observed (data not shown). When compared with other systems that used primers specific to common DNA domains [5,10,13,14], the detection of bovine constituents using our system does not require both restriction enzyme analysis and gel electrophoresis steps; therefore, it helps reduce the time and cost of the operation (i.e. a minute per sample).

4. Qualitative detection of bovine DNAs in feedstuffs

Qualitative detection of bovine DNAs in animal meats and feedstuffs was demonstrated after the presence of DNA in meats and feedstuffs was confirmed according to Wolf et al. [10]. Results of reliable meat species and related samples used in the experiment were shown in Table 1. Among samples tested, three were animal meats, five were raw materials derived from animal blood and bone meals for feedstuffs production, and five were pet foods available on the market. When the detection systems were conducted on the 16 test samples, the system that used the Pth and the 12S rRNA genes as DNA markers could detect bovine DNA and 12S rRNA gene as PCR products of 165 and 115 nt in size, respectively (Fig. 4). The results did not leave any trace of non-specific products even with samples of mixed components as determined by visualization of ethidium bromide stained gel. Although, the detection of Pth gene was based on detection of single copy gene in nuclear genome, the amount of available DNA

Fig. 1. Specificity of PCR system to target Pth gene (A) and 12S rRNA gene (B) among samples. Lane 1 cloned positive DNA; lane 2 negative DNAs; lane 3 non-template control; lane 4 poultry meat DNA; lane 5 ruminant meat DNA and lane 6 porcine meat DNA.
components in feedstuffs was more than enough to be detected by PCR. As a result, this bovine DNA detection could then efficiently detect bovine specific DNA in a similar manner to the one using the cytochrome \( b \) or ATPase8-ATPase6 gene, [10,14] a mitochondrial DNA based detection.

In the electrochemical experiment, the main target study was based on the application of signal transduction induced by the aggregation of target Pth DNA molecule after binding with Hoechst 33258. Typical LSV curves of Hoechst 33258 (20 mM) at 37°C in the absence and the presence of target Pth DNA are shown in Fig. 3. Resulting of anodic peaks to each sample were as described in Table 1. The result of redox current measured was the first demonstration of the aggregation phenomenon of target DNA in the presence of Hoechst 33258 on bovine species detection via Pth gene. Decrease in the anodic peaks from 2.3 \( \mu A \) (Hoechst 33258 solution) and 2.02 \( \mu A \) (non-template control) to 0.88 \( \mu A \) (cloned DNA) was observed in each sample. This corresponded with the amount of DNA detected as evidenced by the sensitivity test (see standard curve on quantitative analysis). The anodic peaks among sample DNAs in Table 1 clearly separated samples into two groups; bovine containing samples having anodic peaks lower than 1.55 \( \mu A \) and non-bovine samples having anodic peaks grater than 1.75 \( \mu A \). This separation agreed well with the PCR results (Fig. 4). Based on the relationship between anodic peaks and DNA concentrations in the study, the threshold of anodic peaks for non-bovine samples could be determined at values above 1.75 \( \mu A \), the level below 85 copies, the corresponding detection limit equalling samples containing less than 0.01 ng DNA described earlier [6]. As a result of the qualitative test, bovine positive samples were detected in ruminant meat (anodic peak at 1.18 \( \mu A \)), 15% protein pet food pellets A (anodic peak at 1.28 \( \mu A \)), 15% protein pet food pellets B (anodic peak at 1.36 \( \mu A \)), 8% protein pet food soup (anodic peak at 1.42 \( \mu A \)), and 17.6% protein pet food pellets (anodic peak at 1.52 \( \mu A \)) (samples no. 5, 12, 13, 14, and 15, respectively). Since the detection via this signal transduction was based on polymerase chain reaction/amplification of the Pth gene, the efficiency of the test in general was then similar to other reliable PCR based methods [14,15]. However, unlike those reported methods using common primers, the detection via the Pth gene with specific primers required no further steps of RFLP analysis and gel documentation. Therefore, this method enables DNA detection in a shorter period of time without laborious steps. Thus, in our experience with quick screening by qualitative tests, aggregation induction by Hoechst 33258 directly on the Pth gene can efficiently discriminate samples with the presence and the absence of bovine DNAs.

Fig. 2. Sensitivity of PCR system specific to Pth gene at various concentrations of DNA templates with; lane M, 100 base pair marker; lane 1–9, 100, 10, 1, 0.1, 0.01, 0.001, 0.0001, and 0.000001 ng DNA template, respectively.

Fig. 3. Characteristics of anodic currents peak (microampere) detected by linear sweep voltammetry of Hoechst 33258 in the absence and the presence of target DNAs.

Fig. 4. Specificity of Pth gene (A) and 12SrRNA gene (B) among 16 samples tested on agarose gel electrophoresis. Lane M marker; lanes 1–3, cloned positive Pth DNA, soybean genome negative DNA, and non-template control; lane 4–6, poultry meat, ruminant meat, and pork meat; lane 7–11, pork meal, porcine protein, porcine meal, poultry by-product meal and nutrition; and lane 12–16, pet food pellet A, pet food pellet B, pet food soup, pet food pellet, and pet food slice, respectively (see also Table 1).
5. Quantitative analysis of bovine contents in feedstuffs

For DNA quantification, the amount of target DNA in samples was calculated based on the measurement of signal transduction induced by aggregation of DNA molecules that were amplified from samples with known copy number ranging from 10^0 to 10^7 copies. To ease the preparation steps, the dilution of the cloned Pth and 12S rRNA fragments in pCRII™ (Invitrogen, USA) was carried out in the same way as one performed in real-time PCR quantification [16]. In the test, simple purification of DNA via Sephadex Column™ was also applied to sample DNAs. This was to reduce unknown traces of PCR inhibitors originated from matrices of feedstuffs that might affect PCR amplification and quantification [17]. This simple purification was not tedious, however. Only requiring little time and cost, it allowed enough quality of DNA samples to be calibrated.

Unlike common real time PCR, in which the detection of DNA signal was based on exonuclease activities of Taq DNA polymerase at 5′ terminus-Tamra dye portion of Taqman probe, measurement of anodic current signals was based on total binding of Hoechst 33258 molecules to the released products and measuring at last step. This was similar to the real time PCR system using SYBR Green™ [18] if considered the last round. Irrespective of target or non-target DNA, and unsaturated or saturated PCR, all DNA products from the reaction after binding went through processes of measurement in the last cycle. However, Hoechst 33258 molecules had less efficiency in binding short double strand DNAs, single strand DNA such as primers, and remaining RNA molecules if any, false signals due to trace of imperfect PCR reaction and parts of dNTP and short primer dimmers would not be delivered. But, if the reactions were either in saturation stage or with some non-specific contamination in the DNA products, wrong signal values would result. We limited non-specific products by adjusting the reaction conditions as shown during qualitative detection. Signal detection during quantification was then manipulated to detect PCR products only before they reached the saturation threshold. In the experiment, the amplification of Pth and 12S rRNA genes at 30 cycles was effective in excluding false signals caused by PCR saturation that could interfere with quantification.

The relationship between a Pth gene dose (concentration via copy numbers/50 μl reaction in log) and an anodic peak (Y: microampere) was then concluded in Fig. 5. Estimation copy number of the Pth gene for each sample was drawn based on the relationship \(Y^{\text{Pth}} = -0.1964X + 2.125\) with \(R^2 = 0.9908\), where \(Y^{\text{Pth}}\) was an anodic peak (microampere) from each sample and \(X\) was a Pth gene concentration (log copy number). Similar calibration was also carried out for 12S rRNA gene with relationship \(Y^{12S} = -0.1823 + 2.0242\) with \(R^2 = 0.9946\) (data not shown). Anodic measurements for both Pth and 12S rRNA genes and their corresponding copy numbers (anti log), calculated from anodic current peak and relationship in term of Pth and 12S equations for ruminant meat (sample no. 5), 15% protein pet food pellets A (sample no. 12), 15% protein pet food pellets B (sample no. 13), 8% protein pet food soup (sample no. 14) and 17.6% protein pet food pellets (sample no. 15) were described in Table 2. Since, it was previously addressed that the Pth gene is single copy per haploid genome [21], the relationship between a target gene and its corresponding cell numbers was then estimated based on the fact that each bovine cell contained two copies of DNAs per diploid (single copy per haploid) whereas total cell numbers of both bovine and non-bovine were estimated from the 12S rRNA genes. The ratios between copy number of Pth genes and total 12S rRNAs in the experiment were used to estimate the bovine content in the samples the same way applied to content determination among genetically modified foods [12,19]. From the estimation, the standard ratio of Pth and 12S rRNA genes in ruminant meat was 0.0239 (1/41.835). This ratio, reflecting the relationship between Pth containing cells (bovine constituents) and total cell numbers, was then employed as the coefficient for bovine content estimation. The bovine content through Pth gene measurement could be estimated from equation; content % = copy of Pth gene × 100/(copy of 12S × 0.0239).

In the experiment, the contents of bovine constituents in four kinds of pet foods were 10.88, 8.76, 6.39, and 2.69% in samples 12–15, respectively. All were below the level of indicated protein contents that appeared on the labels.

The absolute content of bovine constituent among samples was not accurately defined except in sample no. 12 and 13. The estimated value from the experiment indicated that sample no. 12 and 13 contained 10.88 and 8.76% of bovine content, 90.66 and 87.60% accuracy when compared with value 12.00 and 10.00% of defined content, respectively. Samples no.14, and 15, of mixed pet food components, whose defined levels were unavailable, showed the bovine contents of 6.39 and 2.69%, all within the range of the total protein contents addressed in labels.

Accuracy for quantitative estimation of bovine contents could be improved if a total cell numbers could be determined precisely. Since, the copy number of 12S rRNA gene among animal species, commonly used as feedstuff raw materials, was different [20], the calculation of total cell numbers, if feedstuffs were composed of mixed animals and plants ingredients, had to be relied on other fixed copy number of a common gene. Thus,
if the feedstuffs were composed of complex ingredients, using the single copy of Pth and 12S rRNA gene ratio would render only an estimated value.

Since, the relationships between anodic peaks and the Pth and the 12S rRNA genes depend mainly on PCR efficiency, i.e. the concentration of PCR products, which largely depend on sample DNA conditions (source of DNA, purity and so on), and PCR environments, we recommend the construction of standard curve for each measurement.

In this experiment, we had demonstrated how the principle of electrochemical biosensor could be applied to the quantitative and qualitative determinations of bovine constituents in samples through the use of the representative Pth and 12S rRNA gene ratio. In practice, importing feedstuffs and their derivatives into any country was tightly regulated. Only after the confirmation of the absence of bovine constituents by the intensive inspection would items be acceptable for import. Samples with even trace levels of bovine species would be rejected without quantification. For this case, anodic current peak of the Pth gene can be of use as a determination criterion and of use as a one point threshold value in terms of expected microgram contamination. These would all facilitate the processes of testing at best possible convenience.

The measurement using fabricated sensor based on screen print carbon and Hoechst 33258 cost less than US$2.0 per measurement. Although bovine contents could be roughly estimated, we also recommend further studies on 12S rRNA to Pth gene ratio and the ratios among several parts of meat and feedstuff constituents in order to obtain a more precise value of bovine contents within feedstuffs.

| Measurement/content | Ruminant meat (sample 5) | 12% Protein pet food pellets (sample 12) | 15% Protein pet food pellets (sample 13) | 8% Protein pet food soup (sample 14) | 17.6% Protein pet food pellets (sample 15) |
|---------------------|---------------------------|------------------------------------------|------------------------------------------|---------------------------------------|------------------------------------------|
| **Anodic peak (µA)** |                           |                                          |                                          |                                       |                                          |
| Pth gene            | 1.20                      | 1.40                                     | 1.44                                     | 1.51                                  | 1.53                                     |
| 12S rRNA gene       | 0.87                      | 0.88                                     | 0.90                                     | 0.94                                  | 0.89                                     |
| **Copy number (copies)** |                     |                                          |                                          |                                       |                                          |
| Pth gene            | 51,260                    | 4914                                     | 3074                                     | 1353                                  | 1070                                     |
| 12S rRNA gene       | 2,144,480                 | 1,890,024                                | 1,468,108                                | 885,807                               | 1,665,761                                |
| % Total protein     | ND₁                       | 15.00                                    | 15.00                                    | 8.00                                  | 17.60                                    |
| Defined bovine content (%) | 100.00                  | 12.00                                    | 10.00                                    | ND₂                                   | ND₂                                     |
| Estimated bovine content from this test (%) | 100.00                   | 10.88                                    | 8.76                                     | 6.39                                  | 2.69                                     |
| Accuracy (%)        | 100.00                    | 90.66                                    | 87.60                                    | –                                     | –                                        |

ND₁, not determined; ND₂, not defined.

6. Conclusions

We have demonstrated, for the first time, the use of Hoechst 33258 and electrochemical principles for a simple, rapid, and cost effective identification and quantification of bovine DNAs in feedstuffs. By using PCR based detection system that specific to Pth gene of bovine species and 12S rRNA genes, and anodic current peak measurements via linear sweep voltammetry to those DNA products after induced aggregation with Hoechst 33258, target Pth and 12S rRNA genes of the same sample can be detected. Relationships between anodic current peaks among samples and anodic current peaks of known amounts of DNA can be simply compared and employed in both qualitative determination and quantitative estimation of bovine constituents in feedstuffs.

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