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ORIGINAL PAPER

A bioinformatics approach to the development of immunoassays for specified risk material in canned meat products

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Abstract A bioinformatics approach to developing antibodies to specific proteins has been evaluated for the production of antibodies to heat-processed specified risk tissues from ruminants (brain and eye tissue). The approach involved the identification of proteins specific to ruminant tissues by interrogation of the annotation fields within the Swissprot database. These protein sequences were then interrogated for peptide sequences that were unique to the protein. Peptides were selected that met these criteria as close as possible and that were also theoretically resistant to either pepsin or trypsin. The selected peptides were synthesised and used as immunogens to raise monoclonal antibodies. Antibodies specific for the synthetic peptides were raised to half of the selected peptides. These antibodies have each been incorporated into a competitive enzyme-linked immunosorbent assay (ELISA) and shown to be able to detect the heat-processed parent protein after digestion with either pepsin or trypsin. One antibody, specific for alpha crystallin peptide (from bovine eye tissue), was able to detect the peptide in canned meat products spiked with 10% eye tissue. These results, although preliminary in nature, show that bioinformatics in conjunction with enzyme digestion can be used to develop ELISA for proteins in high-temperature processed foods and demonstrate that the approach is worth further study.

Keywords Specified risk material · Bioinformatics · Immunoassay · Heat-processed proteins · Antibody

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Introduction

Since the bovine spongiform encephalopathy (BSE) crisis, there has been a ban on the presence of certain specified risk tissues from ruminants entering the human food chain in order to minimise food-borne exposure risk. These tissues include tonsils, intestines, mesentery, brains, eyes, spinal column, trigerminal ganglia, thymus and spleen.

With the fall in the incidence of BSE the EU commission are reducing BSE control measures,[1] including removing the maximum age of cattle meat entering the food chain, so that specified risk material (SRM) testing of meat products may eventually become the front line in removing SRM from the food chain.

The detection of specified risk material in food is currently limited to the detection of central nervous tissues, e.g. brain and spinal column, and these methods include direct tissue examination, [2] microscopic examination [3], immunoassay [4] or gas chromatography detection of brain cholesterol or specific fatty acids [5, 6]. The immunoassay procedures have been shown to be effective with only lightly cooked meat samples [2], and this is also likely to be the case with more recent methods based on tissue-specific

mRNA [7] but less so with the methods based on methylation of tissue-specific DNA promoters [8].

Immunoassays have long been the only reliable method for the rapid detection of specific proteins in food and clinical samples, e.g. allergens and bacterial toxins. One of the main drawbacks of immunoassay is the specificity of the antibody is limited to the epitopes expressed on the surface of the protein which imposes limits on the specificity the antibody can express for a particular protein. A second drawback to the immunodetection of proteins is that protein tertiary structure becomes disorganised during heating, leading to denaturation, precipitation of some soluble proteins and surface epitopes being hidden in the core of the denatured protein. It has been postulated for some time that exposure of the whole protein sequence for antibody production would significantly improve specificity of the resulting antibodies and possibly overcome the effect of heat denaturation. Also, revealing the whole protein sequence of an antigen could lead to the provision of antibodies with additional specificity such as tissue or species specificity since differences in sequence similarity of the same protein from different species has been used to predict phylogenic relationships, [9]. The rapid growth of DNA sequence information and its translation into protein sequence information has led to the comprehensive public protein sequence databases at http://www.ncbi.nlm.gov/collab. A number of tools to interrogate these protein sequences are freely available on the web, e.g. local alignment of sequences using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), predicted cleavage sites of protein by proteases (http://www.expasy.ch/tools/peptidecutter) and the theoretical determination of the antigenicity of peptide sequences (http://immunax.dfci.harvard.edu/Tools/antigenic.pl).

We have proposed that combining these tools could provide a mechanism to produce theoretical peptide antigens with features that characterise the protein and species from which the peptide was derived. This could ultimately lead to the production of species- or tissue-specific antibodies by in silico investigation of protein sequences. Taking advantage of this opportunity is only hampered by ensuring the target peptide sequence is not locked in the core of the protein in a sample matrix and prevented from binding antibody.

The development of a specific immunoassay based on this approach would depend on being able to cleave the proteins in a sample in a predictable and consistent manner without cleaving the target peptide sequence. Bioinformatics tools are now available which can predict the cleavage sites of proteases and therefore provide the theoretical tool to enable selection of the correct protease to hydrolyse the proteins to peptides in a sample while retaining the target peptide sequence intact.

This paper therefore reports on an evaluation of this theoretical approach to develop immunoassays to specified risk material tissues in canned meat products, opening up the possibility of designing immunoassays for specific proteins irrespective of whether the protein has been heat denatured and specific tissues that can be applied to both native and severely heat-denatured (canned) protein targets.

Materials and methods

Software tools

The Swissprot protein sequence database (www.expasy.ch/sprot/) was interrogated to produce a bovine, ovine, caprine and porcine protein database as well as human and mouse protein sequence database. A database of tissue-specific proteins was produced from these by searching the annotation field within these using the term ‘eye’ or ‘brain’ ‘-specific’. The human and mouse protein databases were similarly searched, and where sequence homology was found for a particular tissue-specific protein, then this protein was also added to the database of tissue-specific (not species-specific) proteins.

The proteins were digested *in silico* using bespoken in-house developed software (http://bioinformatics.csl.gov.uk/) validated against the Expasy peptidecutter program (http://ca.expasy.org/tools/peptidecutter/) and each of the generated peptides was then BLAST searched against the Swissprot database to determine whether the peptide was specific to the parent protein.

In addition, each protein sequence was cut *in silico* into ten overlapping amino acid units, and each ‘10mer’ sequence was BLAST searched against the Swissprot database to determine which regions of the protein sequence were specific to that protein. Protein-specific 10mer units were then evaluated using peptidecutter to determine which were resistant to proteolytic activity.

In-house developed software (http://bioinformatics.csl.gov.uk/) was used to determine those peptides specific to a particular protein, which were least likely to be posttranslationally modified and predicted to be an epitope. The software was capable of determining the most antigenic regions of the peptides.

Synthesis of peptides

Four of the peptides (peptides 1–4) were synthesised commercially by Eurogentec S.A (Belgium). The remaining two peptides (5, 6) were synthesised by Severn Biotech Ltd (UK). All peptides were synthesised to 75% purity (Table 1). The ends of the peptides 1,4 and 5 were ‘closed’ by amidation as the synthesised peptide formed the inner

Table 1 Tissue and species specificity of selected peptide sequences

Peptide specificity	Protein	Specific peptide sequence	Species specificity	Resistant to	Peptide released by protease cleavage
Eye-1	Filensin (BFSP1)	EEGGPPEG	Ruminant	Pepsin	KEEGGPPEGKGEPPEGKGDSVKEEGGPPEGKGDG VKEEGGPPEGKGDGVKEEGGPPEGKGDGVKKEG EPPEGKGEGL
Eye-2	Alpha crystallin	KHNERQDDHG	None	Pepsin	HGKHNERQDDHG VEIHGKHNERQDDHG
Brain-3	Glucose transporter type 3	LWGTEDVAQD	Ruminant	Trypsin/ Pepsin	LWGTEDVAQDIQEMK / WGTEDVAQDIQEMKDESMR
Brain-4	Glial fibrillary acidic protein	EAVASSNM	Ruminant	Pepsin	REIRTQYEAVASSNMHEAEEWYRSKF
Brain-5	Glial fibrillary acidic protein	TQYEAVASSNMHEAEEWYR	Ruminant	Trypsin	TQYEAVASSNMHEAEEWYR
Brain-6	Neurexin	IKEERTPVND	None	Pepsin	NIGTVDISIKEERTPVNDGK

part of a larger theoretical peptide released by the action of the selected protease.

Peptides for immunisation

Peptides were obtained as free peptide and also conjugated to keyhole limpet haemocyanin (KLH) for immunisation using sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC). Conjugation to the peptide was via an additional N-terminal cysteine for all the peptides except peptide 3 where conjugation was via a C-terminal cysteine.

Peptides for antibody screening

Selection of the monoclonal antibodies was carried out using the synthetic peptide conjugated to a carrier other than KLH.

Peptides 1–4 were conjugated to biotin (by Eurogentec) via the additional terminal cysteine using sulfosuccinimidyl 6-[3′-(2-pyridyldithio)-propionamido]hexanoate (sulfo-LC-SPDP) chemistry, while peptides 5 and 6 were conjugated to bovine serum albumin via the peptide terminal cysteine using the same chemistry (carried out by Severn Biotech Ltd)

Antibody production

Two female mice (Balb/c Ola Hsd), 10–12 weeks old, were immunised subcutaneously (sc) with 50 μg of peptide conjugate in 100 μL of phosphate-buffered saline (PBS**)** emulsified with 100 μL of Specol. Booster injections (sc) of 25 μg of peptide conjugate in 100 μL of PBS emulsified with 100 μL of Specol were given at 2-week intervals. The two mice were primed intraperitoneally with 25 μg of peptide in 200 μL of PBS 4–5 days before spleen cell isolation

Selection of monoclonal antibodies

All culture supernatants were screened using enzyme-linked immunosorbent assay (ELISA). For antibodies to peptides 5 and 6, plates were coated with the bovine serum albumin (BSA) peptide conjugate at 5 μg/ml in carbonate buffer pH9.6 overnight at 4 °C then washed in PBS and blocked with 5% milk powder in PBS/0.02% Tween for 2 h at 33 °C. Plates were washed in PBS, then 100 μL tissue culture supernatant, diluted 1:1,000 in PBS/0.02% Tween/0.2% BSA, was added to each well. Quantitation of specific antibody was determined through the addition of rabbit anti-mouse immunoglobulin G (IgG)–alkaline phosphatase conjugate.

For peptides 1–4, ELISA plates (Nunc Maxisorp®) were coated with Neutravidin (Pierce) at 5 μg/mL, then blocked with fish gelatine and then coated with the biotinylated peptides at 2 ng/mL in carbonate buffer pH9.6. Tissue

culture supernatant containing monoclonal antibodies, diluted 1:1,000 in PBS was added and quantitation of specific antibody determined through the addition of rabbit anti-mouse immunoglobulin G (IgG)–horseradish peroxidase conjugate.

For the determination of the 50% inhibition concentration (IC 50) of the synthetic peptides, a competitive ELISA was developed. Purified antibody and unbound peptide were added to ELISA plates coated with biotinylated peptide as described above. Quantitation of the bound antibody was via the addition of rabbit anti-mouse IgG horseradish peroxidase or alkaline phosphatase conjugate.

All selected monoclonal antibodies (isotype IgG) were purified on a HiTrap® Protein G column (GE Healthcare) from their tissue culture supernatants.

Raw materials and reagents

Purified glial fibrillary acidic protein (GFAP; bovine spinal cord) in 6 M urea and 2 mM dithiothreitol was purchased from American Research Products (Belmont, MA USA). Purified alpha crystallin (from bovine eye lens) was purchased from Sigma (product code C4163).

Brain and eye tissues from cow, pig, sheep and goat were obtained from the Veterinary Laboratory Agency, Thirsk, UK.

Canning of meat samples

All heat-processed samples were homogenised as raw tissue then heat-treated in a canning retort at 121 °C for 10 min.

Tissue extracts

Tissue samples were homogenised twice in nine volumes (*w*/*v*) of acetone and the solids recovered by centrifugation at 2,000×*g* for 10 min. The solids were then extracted with two volumes (*w*/*v*) of *n*-butanol by grinding the slurry with a mortar and pestle. The solid residue was recovered by centrifugation, washed in hexane and allowed to air dry before grinding with a mortar and pestle until the powder passed a 0.1-mm sieve. The sieved powder was stored at −20 °C.

Five millilitre of PBS was added to 0.5 g of the dried samples and vortexed vigorously for 5 min. Samples were centrifuged at 2,000×*g* for 10 min and the supernatant removed. This supernatant was used in subsequent assays both in plate-trapped ELISAs and in blotting studies.

Extracts of 13 tissues were prepared for cross-reactivity studies: Bovine kidney, liver, ileum, spleen and eye; porcine kidney, liver, ileum, eye and brain; and ovine ileum, eye and heart.

Proteolytic digestion of samples

Pepsin

One milligram freeze-dried sample ("Tissue extracts" above) was suspended in 0.966 mL glycine/HCl buffer (0.155 M, pH1.3), and 4 µL pepsin solution (Sigma Chemical Co. product P7000; 25 mg/ml in MilliQ water) was added, mixed carefully and the sample incubated overnight at 37 °C in a shaking water bath. After digestion, 30 µL 5 M sodium hydroxide was added to stop proteolysis.

Meat samples

One gram meat sample (not dried) was suspended in 8.7 mL glycine/HCl (0.172 M, pH1.3) and 1 mL pepsin solution added (100 mg/mL in water). Samples were incubated overnight at 37 °C in a shaking water bath. After digestion, 30 µL of 5 M sodium hydroxide was added to 970 µL digested sample to return the sample to between pH 7 and 8.

Trypsin

Ten milligrams of powdered sample was incubated with 0.4 mL of 25 mM sodium hydroxide for 30 min at 37 °C with shaking. The sample was then adjusted to pH7.5 with 0.1 mL 1 M HCl, and 25 µL 1 M Tris buffer pH7.5, 25 µL of 2%(*w*/*v*) sodium dodecyl sulphate (SDS) and 25 µL of 40 mM tris(2-carboxyethyl)phosphine were then added.

The sample was incubated with shaking for 30 min at 37 °C then diluted 1+1 with 200 mM Tris buffer pH8.0

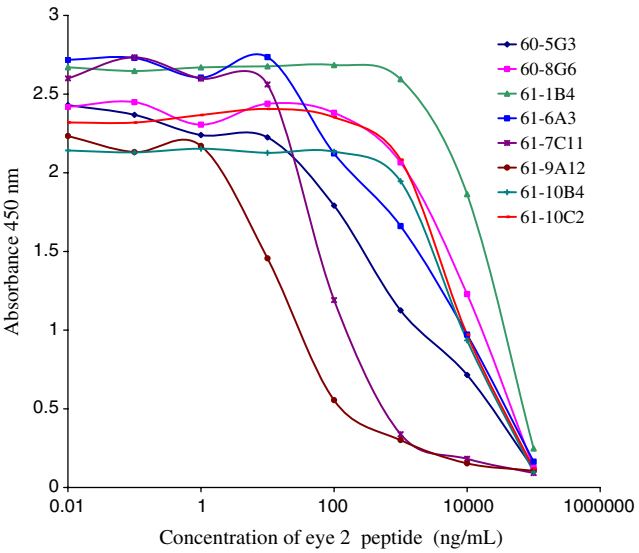

Fig. 1 Inhibition curves for the eight selected eye-2 monoclonal antibodies (antibodies inhibited using free eye-2 peptide)

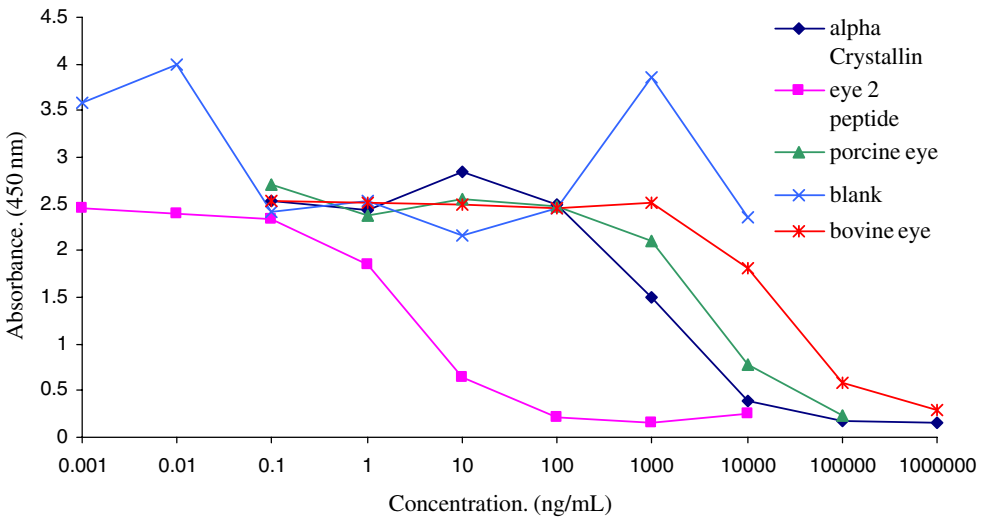

Fig. 2 Detection of eye tissue using eye-2 antibody and pepsin digest of eye lens extract in competitive ELISA with plate-trapped peptide (coated at 2 ng/mL)

and 1 mg trypsin (Trypsin Proteomics Grade; T 6567, Sigma) added and incubated overnight at 37°C with vigorous shaking. After incubation, the sample was made 2 mM with phenylmethane sulphonyl fluoride in *n*-propanol and heated to 100°C for 10 min before centrifugation to clarify.

Results

Selection of peptides

Two peptides from the sequence of the brain-specific GFAP protein were identified as likely to be resistant to proteolysis either by pepsin or trypsin and their sequences shown by BLAST searching against Swissprot (http://blast.ncbi.nlm.nih.gov) to be specific to the protein from ruminant species only (Table 1). Single peptides were also identified from each of the two brain-specific proteins, glucose transporter type 3 and neurexin. The sequence of the glucose transporter 3 peptide was also shown through BLAST searching to be specific to ruminant species. Both peptides were shown to be likely to be resistant to trypsin cleavage.

Two peptides from the eye-specific proteins alpha crystallin and filensin were also identified by the in-house software to be likely antigenic peptides specific for eye tissue. The filensin peptide was shown by BLAST searching Swissprot to have a sequence specific to ruminant species; however, the alpha crystallin peptide was shown to have no species specificity at all.

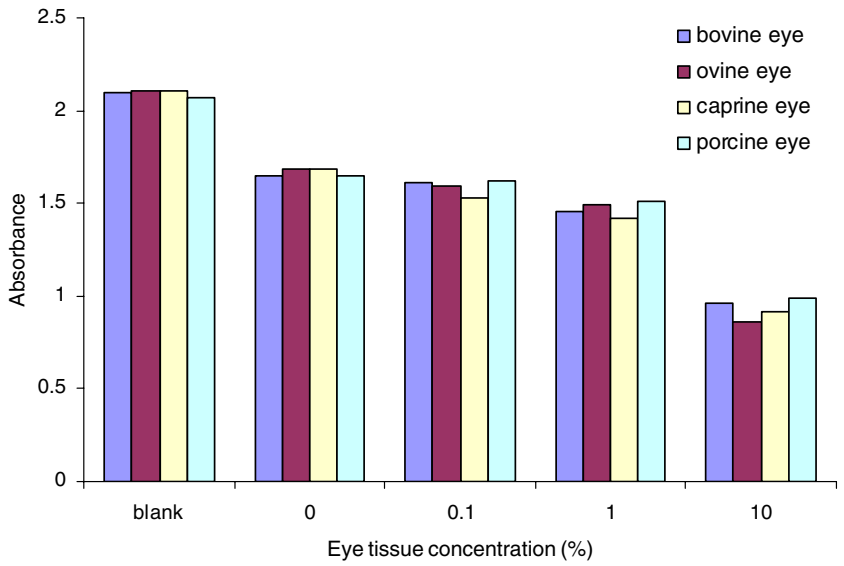

Fig. 3 Detection of eye tissue of different species in canned meat using eye-2 antibody and pepsin digests of the tissue in a competitive ELISA with plate-trapped peptide

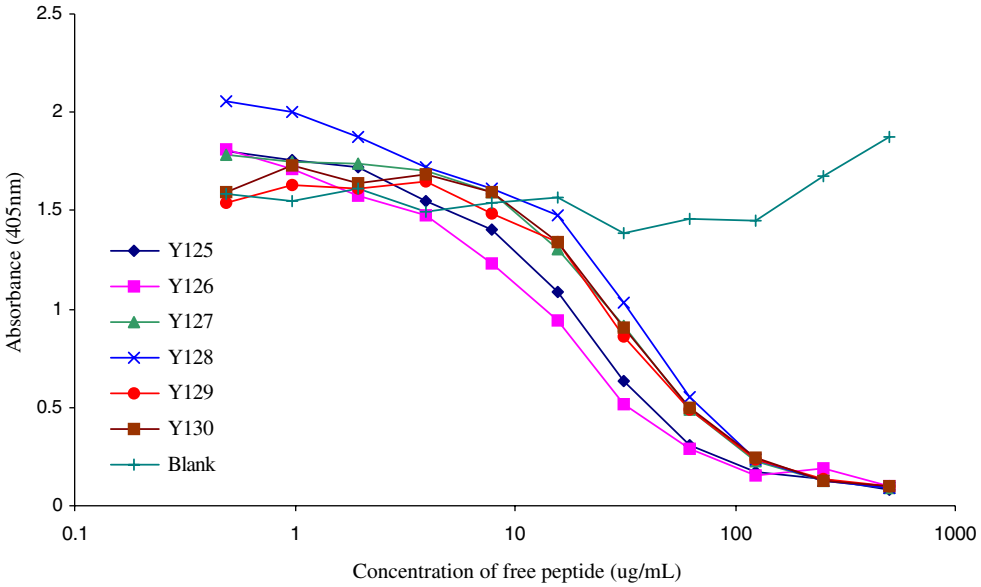

Fig. 4 Inhibition curves for the six selected brain-4 monoclonal antibodies (antibodies inhibited using free brain-4 peptide)

Antibody characterisation and assay development

No monoclonal antibodies to the target peptides were identified for three of the peptides, brain-5 peptide, (the 16mer peptide to the brain protein GFAP), brain-3 peptide (10mer peptide to Glucose transporter type 3) and eye-1 (8mer to filensin).

Eight monoclonal antibodies were isolated for peptide eye-2. A preliminary screening in competitive ELISA confirmed the affinity of the antibodies for the target peptide ranged from ca. 10 ng/mL to ca. 50 µg/mL (Fig. 1) with no cross-reactivity with the other biotinylated peptides tested. For subsequent experiments, MAb 6 (61-9A12), displaying the highest affinity, was selected.

Studies using pepsin on the synthetic peptide confirmed that the peptide was not digested. In competitive ELISA, undigested alpha crystallin was not detected by MAb 6, as opposed to pepsin digested alpha crystallin. This clearly showed that enzyme digestion was essential for binding of MAb6 Pepsin digests of bovine, and porcine cooked eye tissue was also detected by MAb6 (Fig. 2). The IC 50 values for purified alpha crystallin were approximately 1–2 µg/mL compared with 2 ng/mL for the free peptide. This was approximately 50 times less sensitive for the protein digest than the synthetic peptide when compared on a molar ratio (molecular mass of alpha crystallin 19.7 kDa compared with the molecular mass of the peptide of 1338 Da). The antibody did not detect pepsin digests of the ten other tissues used in the-cross reactivity studies.

In tests carried out on canned meat products containing homogenised eye tissue of different species, the assay readily detected the addition of 10% eye tissue to the meat (Fig. 3). In the assay, the meat digests were diluted to a meat concentration of 10 mg/mL. The difference in absorbance between the blank and the meat without added eye tissue indicated some inhibition due to matrix effects which was assumed to be due to the high protein and fat concentrations in the extracts. The detection limit of the developed assay was between 1% and 10% eye.

Six monoclonal antibodies were selected for peptide brain-4. These were shown not to cross-react with KLH. Competitive ELISA confirmed the affinity of two of the antibodies for the synthetic peptide with IC 50 values calculated at 3.9 and 18.4 µg/mL (Fig. 4). Considering the molecular mass of the peptide (807 Da) and the molecular mass of GFAP (54 kDa), the 3.9 µg/mL IC 50 value for the synthetic peptide corresponds to 256 µg/mL for the whole GFAP protein. The antibodies were unable to detect native

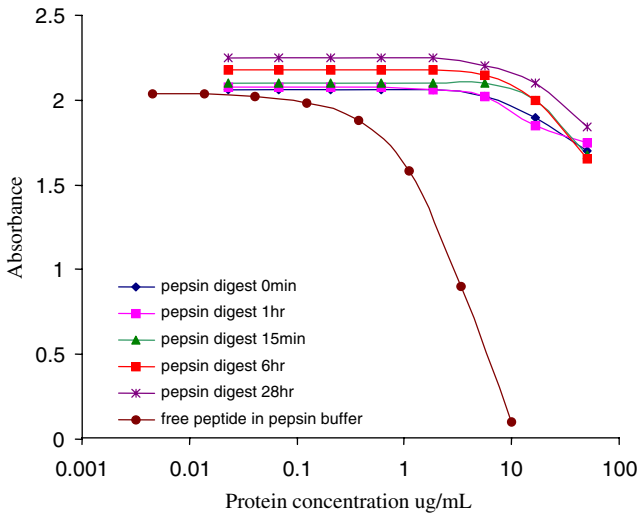

Fig. 5 Effect of pepsin digestion of GFAP on the binding of brain-4 MAb (competitive ELISA)

extracts of bovine brain by immunoblotting but could detect 6 M urea extracts of bovine and porcine brain and GFAP suggesting the antibody had no species specificity.

Studies using pepsin and trypsin on the synthetic peptide confirmed that the peptide was not digested by either of these proteases, and competitive ELISA using pepsin digests of GFAP showed the digests were detected by the antibody at high concentrations of GFAP used (50 μg/mL; Fig. 5) This is in line with the low affinity of the antibodies for the synthetic peptides, suggesting the antibody had specificity for the GFAP protein target but with an affinity corresponding to concentrations higher than were present in extracts of brain tissue.

Four monoclonal antibodies were isolated for the brain-5 peptide that showed no cross-reactivity with KLH. These had IC 50 values for the free brain-5 peptide of between 0.1 and 1.0 μg/mL. However, in studies with brain tissue digests, the antibodies showed no species specificity. A comparison of the sensitivity of a commercial antibody for GFAP (supplied by R Biopharm AG) revealed that antibodies to both the brain-4 and brain-5 peptides were significantly less sensitive towards an SDS extract of cooked brain tissue than the commercial antibody suggesting these monoclonals had little to offer over the existing commercial brain tissue-specific antibody.

Discussion and conclusions

The successful production of an ELISA for cooked eye tissue in canned meat, based on the identification of a short protease-resistant peptide using bioinformatic tools, has demonstrated that an *in silico* approach to generating immunoassays for cooked proteins is credible. The data are, however, of a preliminary nature and demonstrate that the approach is worth being further developed.

The ELISA for the digest of the alpha crystallin protein was approximately 50-fold less sensitive than for the synthetic peptide, probably reflecting the degree of digestion of the parent protein. However in the limited specificity study, the ELISA was specific to eye tissue, and therefore, the theoretical approach was valid for tissue specificity.

The assay in its present form can detect only 1–10% eye tissue in a canned meat matrix, which is too insensitive to be of any real commercial value. There is, however, potential to increase the sensitivity of the assay through solvent extraction to remove interfering materials as well as post digestion enrichment of selected peptides using high throughput solid-phase extraction.

The inability of the approach to produce any species-specific antibodies could not be explained. None of the amino acid residues in those sequences that were species specific were particularly labile and, in the case of the peptide sequences derived from the brain protein GFAP, the sequences for a range of animals were in the databases, and so sequence incompatibility between the species of interest could be confirmed. Further studies into generating species-specific peptides is clearly required.

The three peptides that did not lead to the isolation of specific monoclonal antibodies had nothing particular in common. Two of these had C-terminal amidation, the same as peptide brain-4 that resulted in a specific brain monoclonal antibody with low avidity. The successful peptide eye-2 antibody had cysteine attached at the N terminus as did peptides eye-1, -2 and brain-4 and -5 peptides.

In conclusion, this study has shown that the *in silico* identification and synthesis of short protease-resistant peptides, unique to tissue-specific proteins, can be used as a means of developing immunoassays in conjunction with protease digestion, for animal tissues in cooked meat, even after high-temperature processing.

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