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Pepsin Digestion of Antibodies to Produce Functional Antigen-Binding Fragments (Fab): A Scientific Fantasy?

Penelope J. Gauci and
Malcolm R. Alderton

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Penelope J Gauci and Malcolm R Alderton

Combatant Protection and Nutrition Branch
Aeronautical and Maritime Research Laboratory

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ABSTRACT

A pepsin antibody fragmentation procedure was trialed and optimised. The procedure used was a modified version of that described in Current Protocols in Immunology. Pilot digestions using a range of pepsin concentrations and incubation times were completed at pH 4.0 and pH 4.5. The digestion products were examined by Phastgel electrophoresis, and the combination of pepsin concentration, incubation time and pH that provided the most efficient digestion of the antibody into dimeric antigen-binding fragments ($F(ab')_2$) was then used in the large-scale digestion of the antibody. Monomeric antigen binding fragments (Fab) were obtained through reduction of $F(ab')_2$ using 2-mercaptoethanol. In this study Fab from a mouse polyclonal anti-ricin antibody and a sheep polyclonal anti-*Bacillus anthracis* antibody generated with the optimised procedure were comparable to the whole molecule when used as the capture antibody in an ELISA. Nevertheless, it was noted that, in spite of meticulous optimisation of the digestion process it can be fraught with problems.

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Pepsin Digestion of Antibodies to Produce Functional Antigen-Binding Fragments (Fab): A Scientific Fantasy?

Executive Summary

An optimised procedure for the efficient separation of antibodies into functional antigen-binding fragments (Fab) using pepsin digestion and 2-mercaptoethanol reduction was investigated. The production of these fragments is an essential step in the development of an ion channel switch (ICS) biosensor for the detection of the plant toxin ricin, and the causative agents of anthrax, plague and Q fever. Biosensors for the detection of these potential BW agents will be of considerable benefit to the ADF. The affinity of the Fab for their antigen is critical for the development of sensitive ICS biosensors, and the affinity can be diminished or eliminated if the concentration of pepsin or the reducing agent is too high, or the incubation is too long.

When the concentration of pepsin to antibody in the digestion is too high the enzyme not only digests the antibody into dimeric ($F(ab')_2$) and Fc fragments, but attacks the antigen-binding site on the Fab. The effect of the enzyme on the antigen-binding site cannot be estimated prior to digestion and testing. For example, dimeric antibody fragments ($F(ab')_2$) were produced for each of the four antibodies described in this report, but only two of these were immunoreactive in an ELISA. Similarly, when the concentration of 2-mercaptoethanol is too high, and or, the incubation is too long, the integrity of the antigen binding site can be compromised and the antigen-antibody affinity reduced or eliminated.

In assessing the antigen affinity of the Fab fragments generated in the pepsin digestion and 2-mercaptoethanol reduction, researchers need to ensure that the assessment protocol is well planned and optimised. In this study, for example, when polyclonal anti-ricin $F(ab')_2$ were used as the capture antibody in an ELISA they had a substantial reduction in affinity for the antigen when compared with whole anti-ricin antibodies. However, when these $F(ab')_2$ were assessed in a direct ELISA in which ricin was adsorbed to the microtitre plate the reduction in antibody affinity was not nearly as pronounced.

Despite the problems identified above with generating functional Fab via proteolytic digestion with pepsin, a protocol was developed and high affinity Fab produced for use in the Australian Membrane and Biotechnology Research Institute (AMBRI) ICS biosensor, albeit with a fifty percent success rate.

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Penny Whitehead graduated from the University of Melbourne with a B Sc in 1992. She joined the Department of Applied Biology amd Biotechnology, RMIT University as a Technical Assistant in 1992, before being promoted to Technical Officer in 1993. Her work at RMIT included production and purification of polyclonal and monoclonal antibodies, antigen purification, chromatography, vaccine production and animal trials for vaccine efficacy testing. She joined AMRL in 1998 and has worked predominantly on detection of biological materials, including developing antibodies for use in the Australian Membrane and Biotechnology Research Institute (AMBRI) ion channel switch (ICS) biosensor.

Malcolm R Alderton
Combatant Protection and Nutrition Branch

Malcolm Alderton graduated from Monash University with a B Sc (Hons) in 1980 and a PhD (Immunology) in 1984. In 1994 he graduated with a Grad Dip Ed from RMIT and in 2000 he graduated with a Grad Cert Man from Flinders University. In his first postdoctoral position with Biotechnology Australia he worked on the coccidiosis project at CSIRO Animal Health (Parkville). He moved to RMIT University in 1988 and worked as a Research Scientist and Lecturer before joining AMRL in 1995 as a Research Scientist. In January 1997 he was promoted to Senior Research Scientist and in 1998 became a Principal Investigator in a collaboration with AMBRI Pty Ltd to develop the AMBRI ion channel switch (ICS) biosensor for defence applications. In July 2000 he became the Task Manager for Biomedical Defence Against Biological Warfare (BW) Agents, and March 2001 he started a 12 month sabbatical with the CSIRO Health Sciences and Nutrition Division.

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1. Introduction

The structure of a monomeric immunoglobulin molecule is provided in Fig 1. Briefly, it consists of four amino acid chains - two identical heavy chains and two identical light chains - connected by several intra- and inter-chain disulfide bridges. Each molecule has two identical antigen binding sites each consisting of the variable domains of a heavy and light chain at the amino end of the molecule. The amino acids in hypervariable regions or complementarity determining regions (CDRs) within the binding site govern its shape, and consequently determine the antigen it recognises. The hinge region, which contains the interchain disulfide bridges that separates the antigen binding sites from the Fc (crystalline fragment) segment, is the region preferentially targeted by proteolytic enzymes.

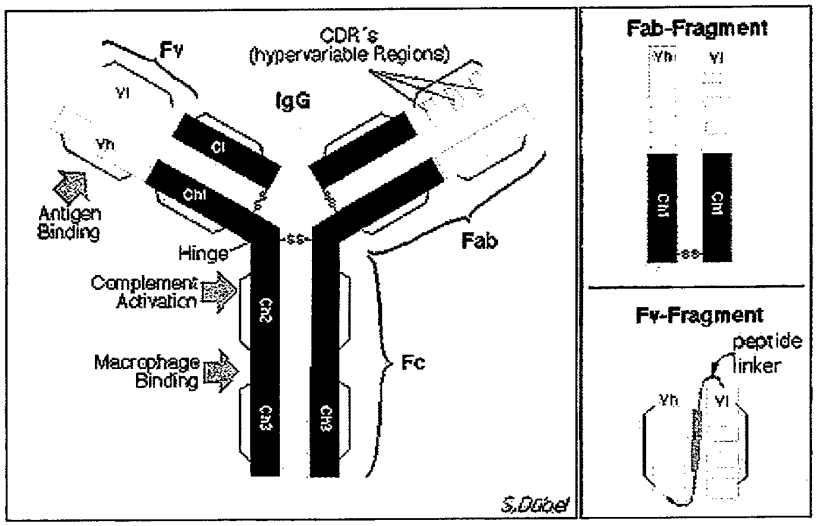

Figure 1 Monomeric antibody unit and antigen-binding fragments

The removal of the Fc portion of an antibody significantly reduces, or eliminates, its interaction with immune cells and decreases non-specific binding (Lamoyi, 1986). This is a prerequisite of many immunoassays and biosensors, including the Australian Membrane and Biotechnology Research Institute (AMBRI) ICS biosensor. The separation of the dimeric antigen binding site fragments ($F(ab')_2$) into Fab is achieved by incubating dimers with a reducing agent such as 2-mercaptoethanol.

Antibodies of the immunoglobulin G (IgG) class have been identified as the most suitable antibodies for use in the ICS biosensor (P.Osman, personal correspondence). This class of antibodies generally have a higher affinity for the antigen than other immunoglobulin classes. Furthermore, mouse monoclonal antibodies (Mab) of the IgG_1 subclass are the preferred antibody for use in the ICS biosensor, and chymopapain the chosen proteolytic enzyme as it preferentially cleaves IgG_1 molecules. The digestion of IgG_1 Mab with chymopapain, and indeed other proteolytic enzymes, generally provides a substantial yield of strongly reactive $F(ab')_2$. Unfortunately, not all antibodies are produced in mice, nor are they always of the IgG_1 subclass. Consequently, a proteolytic enzyme with the capacity to cleave $F(ab')_2$ from all antibody classes, and in particular the IgG subclasses, is needed. The proteolytic enzyme pepsin, rightly or wrongly, has assumed this mantle. However, a major problem identified with pepsin digestion of antibodies is that it does not always produce immunoreactive fragments (Milenic *et al.*, 1989).

This report describes attempts to optimise pepsin digestion of antibodies to produce a method for generating high quality Fab from a mouse Mab, and sheep, goat and mouse polyclonal antibodies.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Pepsin, Trizma® Base, Iodoacetamide, Ricin, and PBS were purchased from Sigma-Aldrich, Castle Hill, NSW. Sodium acetate (AnalaR) was acquired from BDH. The 2-mercaptoethanol was purchased from Riedel-de Haën.
Yersinia pestis antigen was purchased from CSL Pty Ltd, and *Bacillus anthracis* antigen was purchased from Colorado Serum Company, Denver, USA.

2.1.2 Antibodies

Dr Kuchel of the South Australia Institute for Medical and Veterinary Science produced a sheep polyclonal anti-*Bacillus anthracis* antibody under contract.
The Defence Research Establishment Suffield (DRES) in Canada provided a goat polyclonal anti-*Yersinia pestis*.
Anti Ricin monoclonal and polyclonal antibodies were produced at DSTO.
Antibodies conjugated with alkaline phosphatase for the ELISAs were purchased from Sigma Aldrich.

2.2 Methods

2.2.1 Ricin antibody preparation

2.2.1.1 Polyclonal anti –ricin antibodies

Polyclonal ascites to ricin were produced in female Balb/c mice which were 10 weeks old at the start of the inoculation program. Each mouse received 0.25 ml of pristane, followed by 4 or 5 inoculations of 1-3 μg of ricin peptide A, or the whole ricin molecule, in a Freund's Incomplete Adjuvant (FIA) emulsion. The administration of the antigens began 4 to 5 days after the pristane injection and was given at two-week intervals. Four to seven days after the final antigen injection the mice received 10^7 Sp/2 myeloma cells. All injections were given intraperitoneally and the ascites fluid was drained from the peritoneal cavity of the mice 10 to 14 days after the administration of the myeloma cells. The ascites were centrifuged at 1000 x *g* for 7 min to remove cells and protein debris.

2.2.1.2 Monoclonal anti-ricin antibodies

Monoclonal anti-ricin antibodies were produced using spleens from female Balb/c mice that received the same inoculation program as described above.

2.2.2 Purification of antibodies

The IgG fractions of polyclonal anti-ricin antibodies were obtained using Protein G columns (Pharmacia) according to the manufacturer's instructions. Protein concentrations were estimated using a modified version of the Lowry method (Markwell *et al.*, 1978).

The purity of the IgG fractions were visually determined using Phastgel electrophoresis and Coomassie blue staining.

2.2.3 Pilot pepsin digestions

Small-scale pilot digestions were conducted for each antibody to identify the most appropriate conditions for the large-scale production of $F(ab')_2$. In these experiments the optimum concentration of pepsin, digestion time and pH were examined.

Prior to digestion the antibody was transferred into 0.2M sodium acetate buffer (pH 4.0) by dialysis or the use of Centricons (Amicon). The antibody

concentration was then adjusted to 2 mg/ml. In the initial experiments 0.2M sodium acetate buffer was also used at pH 4.5, but at this pH the proteolytic activity of the pepsin was greatly reduced. The antibody digestion was completed using an equal volume of a pepsin solution prepared in the same buffer and providing a final w/w ratio of pepsin to antibody ranging from 1:20 to 1:1000. The resultant enzyme/antibody mixture was incubated at 37°C for 1, 2, 4, 6 or 24 hours. At the designated time, 2M Trizma® Base was added to adjust the pH to neutral to stop the reaction. Finally, the products of each reaction were analysed on a Phastgel to determine the best digestion conditions, which were then used to produce $F(ab')_2$ on a large scale.

2.2.4 Purification of dimeric antigen binding site fragments

Following the large-scale digestion, the antibody fragments were dialysed overnight in phosphate buffered saline (PBS) and the volume reduced to approximately 1 ml. The solution containing the antibody fragments was applied to two Sephadex 75 columns (Pharmacia) arranged in series to isolate the $F(ab')_2$ from the other digestion products. The relevant fractions were then pooled and concentrated to approximately 1 mg/ml.

2.2.5 2-Mercaptoethanol reduction of dimeric antigen binding site fragments

The pilot reduction of the dimer to Fab was performed using 5 to 35mM solutions of 2-mercaptoethanol (2ME) in an antibody to 2ME ratio of 10:1 (v/v), over a period of 1 to 3 hours. The reaction was stopped at the designated time with the addition of 0.2M iodoacetamide. The resultant products were analysed on a Phastgel to determine the best reduction conditions, which were then used to complete a large scale reduction of the dimer. At the end of the large-scale reduction the Fab were separated from the 2ME on a series of desalting columns (Pharmacia) instead of stopping the reaction with the iodoacetamide.

3. Results and Discussion

Traditionally, pepsin has been the enzyme used to produce dimeric antibody fragments ($F(ab')_2$) from antibodies produced in numerous animal species (Nisonoff *et al.*, 1975). The $F(ab')_2$ can be easily reduced to Fab if desired. This procedure was used to produce Fab for the AMBRI ICS biosensor as it preserves the sulfhydryl groups (of the reduced disulfide bridges) required for efficient biotinylation of the antibody fragments to be incorporated into the biosensor.

The effectiveness of generating $F(ab')_2$ with pepsin was examined using the methods described above on a sheep anti-*Bacillus anthracis* polyclonal antibody, a goat anti-*Yersinia pestis* polyclonal antibody, a mouse anti-ricin polyclonal antibody and a mouse anti-ricin monoclonal antibody.

3.1 The sheep anti-*Bacillus anthracis* polyclonal antibody

Fig 2 details the results of a capture ELISA that compares the antigen affinity of the intact sheep anti-*Bacillus anthracis* polyclonal antibody and its dimeric antigen binding fragments. In this pilot study the digestion incubation times of 1, 2, 4 and 24 hours had little effect on the activity of the resultant fragments, which were comparable to the intact antibody. Similarly, the enzyme to antibody ratio (E:A) of 1 to 100 used in these experiments did not affect the antigen affinity of the $F(ab')_2$. A large-scale production of $F(ab')_2$ from this antibody using an E:A of 1 to 100 in a 4 hour incubation displayed high affinity for the antigen. 2-Mercaptoethanol reduction of the $F(ab')_2$ into Fab did not affect antigen affinity.

Clearly, the procedure which involved a pepsin digestion and subsequent reduction step, was effective in producing high affinity Fab from the IgG fraction of this sheep antibody.

3.2 The goat anti-*Yersinia pestis* polyclonal antibody

The pilot study for the goat anti-*Yersinia pestis* antibody indicated a 6 hour incubation with an E:A of 1 to 100 and a 1 hour incubation with 15mM 2-mercaptoethanol was optimum for the Fab production (data not shown). Fig 3 shows the comparative antigen affinity of the whole molecule and the Fab in a capture ELISA. These results show there has been a substantial and unacceptable reduction in the antigen affinity of the Fab. Clearly, the pepsin digestion and/or the 2-mercaptoethanol reduction has had a deleterious effect.

After further investigation, the pepsin digestion was shown to be largely responsible for the substantial loss in antigen affinity. The mercaptoethanol reduction had little if any effect on the affinity of the Fab for the antigen (data not shown). However, why the same digestion procedure should be effective for the sheep antibody described above and ineffective for this antibody from a closely related species remains an enigma.

One possible explanation relates to the variability in the hinge region of the antibody. The amino acid composition and location of disulfide bonds in the hinge region can vary markedly between antibodies. Such variations could

determine the resistance of the antibody to the enzyme and/or the regions of the antibody digested by the enzyme. A significant loss in antigen affinity like that observed for the antibody in this study would occur if the antigen-binding site became a site of action for the enzyme.

3.3 The mouse anti-ricin polyclonal antibody

A comparison of the antigen affinity of a polyclonal mouse anti-ricin antibody and the $F(ab')_2$ generated in a pilot study using a 24 hour incubation and an E:A of 1 to 120 (A) and 1 to 1000 (B) is presented in Fig 4. The results presented indicate that the $F(ab')_2$ had reduced activity when compared to the whole antibody, albeit not great enough to suggest the Fab could not be used effectively in a capture immunoassay. The studies also revealed that the enzyme to antibody ratios used had little affect on affinity of the $F(ab')_2$ for the antigen. Nevertheless, the $F(ab')_2$ generated using the higher ratio displayed marginally higher affinity than those produced using the lower ratio at the lower concentrations of ricin. The pepsin digestion of this polyclonal mouse anti-ricin antibody, like the digestion of the sheep anti-*B.anthracis* antibody, was effective in producing high affinity dimeric fragments.

These antibody fragments also provide an example of the importance of choosing the most effective method for assessing the affinity of $F(ab')_2$ for the antigen. When these antibody fragments were assessed in an ELISA using ricin adsorbed to the microtitre plate there was a substantial reduction in their affinity for the antigen when compared with whole anti-ricin antibodies (data not shown). However, when they were assessed in a capture ELISA using anti-ricin antibodies adsorbed to the microtitre plate the reduction in antibody affinity was not nearly as pronounced.

3.4 The mouse anti-ricin monoclonal antibody

Isotyping of this mouse anti-ricin monoclonal antibody identified it as an IgG_1 antibody.

This final example of $F(ab')_2$ production not only identifies problems with the digestion process but also confirms the need for careful selection of the immunoassay used in antigen affinity comparisons. Fig 5 provides a comparison of antigen affinities for a mouse anti-ricin monoclonal antibody and $F(ab')_2$ using a capture ELISA (A) and a direct ELISA (B). The $F(ab')_2$ used in these investigations were produced in a pilot study using an E:A of 1 to 100 and 1, 2, 4, 6 and 24 hour incubations. The results of the capture ELISA using the mouse anti-ricin monoclonal antibody and $F(ab')_2$ as a capture antibody (Fig

5A) suggest the monoclonal antibody and the $F(ab')_2$ do not recognise the antigen. However, the ELISA where the ricin is bound directly to a microtitre plate (Fig 5B) indicated that the intact molecule has an affinity for the ricin but the $F(ab')_2$ do not. The loss of antigen affinity was rapid with a one-hour incubation sufficient for the loss of antigen recognition.
The results in 3.3 and 3.4 are interesting in light of debate over the efficiency of pepsin digestion of mouse antibodies into $F(ab')_2$. The available information suggests that there is considerable variation in the production of dimeric antibody fragments from the different mouse IgG subclasses. Parham *et al.*, (1982) suggested that murine IgGs, especially IgG_1, were highly resistant to pepsin digestion. In fact, the traditional pepsin digestion procedure only worked well with mouse IgG_{2a} and IgG_{2b}, though relatively high pepsin to antibody ratio was required. Burton (1985) supported this view stating that the mouse IgG_1 heavy chain lacked the pepsin digestion site – leu 234 – that is highly conserved in humans and other species. Mariani *et al.*, (1991) confirmed that mouse IgG_1 monoclonal antibodies displayed variable resistance to pepsin digestion. The enzyme was unable to digest some antibodies, caused others to crumble into heterogenous fragments, and produced small inactive peptide fragments from others.

In contrast, Lamoyi and Nisonoff (1983) indicted that moderate to excellent yields of $F(ab')_2$ could be obtained from mouse IgG_1, IgG_{2a} and IgG_3 antibodies. They obtained similar digestion patterns on SDS-polyacrylamide gels, and very good dimeric fragment yields for three IgG_1 monoclonal antibodies. A fourth IgG_1 monoclonal antibody derived from the fusion of cells from the same mouse was rapidly destroyed by digestion with pepsin under the same conditions. They also confirmed the finding of Gorini *et al.*, (1969) that dimeric fragments of IgG_{2b} cannot be produced by pepsin digestion, a finding that was later verified by Parham (1983, 1986) and Kim *et al.*, (1994).

In the digestion of the mouse polyclonal and monoclonal antibodies with pepsin described above there were no problems in generating the $F(ab')_2$, but immunoreactivity in the fragments derived from the monoclonal antibody was completely lost. These results do not support the claim that mouse antibodies and in particular IgG_1 monoclonal antibodies are highly resistant to pepsin digestion.

Mariani *et al.*, (1991) suggested there was a microheterogeneity in mouse monoclonal antibodies, and this could explain some of the difficulties associated with peptic digestion of mouse monoclonal antibodies, including the anti-ricin monoclonal used in this study. A large number of heavy chain

variants in the monoclonal antibody solution could mean the antibodies would digest at differing rates, and result in no substantial quantity of immunoreactive dimers being produced.

The disconcerting reduction in, or loss of, antigen affinity in the dimeric antibody fragments is well documented in the literature. Yamaguchi *et al.*, (1995) suggested that IgGs are not stable under pepsin digestion conditions, and in particular, the low pH required can irreversibly reduce the antigen-binding affinity of the $F(ab')_2$ obtained.

4. Conclusions

Variations in the hinge region of individual antibodies, and the suggested microheterogeneity of mouse monoclonal antibodies (Mariani *et al.*, 1991), indicate that the optimum conditions for pepsin digestion need to be determined for each individual antibody. The work described in this report shows that although this is a logical approach to the problem it does not guarantee the production of Fab with an antigen-affinity suitable for use as a therapeutic agent or in an immunoassay. The literature describing the production of $F(ab')_2$ using proteolytic digestion confirms the process provides no guarantees regardless of the digestion method used.

This study also identifies the importance of selecting the most appropriate method of assessing the antigen-affinity of the antibody. Clearly it is critical to have an assay that provides a true assessment of affinity of the antibody. It would be preferable to have more than one method of assessment to ensure a truly indicative result has been obtained.

This fragmentation and digestion method was successful in generating functional Fab for a sheep anti-*Bacillus anthracis* antibody and a mouse anti-ricin polyclonal antibody. However, it was unsuccessful in the case of a goat anti-*Yersinia pestis* polyclonal and a mouse anti-ricin monoclonal antibody. The fifty per cent success rate in generating functional Fab described in this study may not be indicative of proteolytic digestion of antibodies but it does highlight the unpredictability of the process. Therefore, is the pepsin digestion of all antibodies to produce functional antigen binding site fragments a scientific fantasy?

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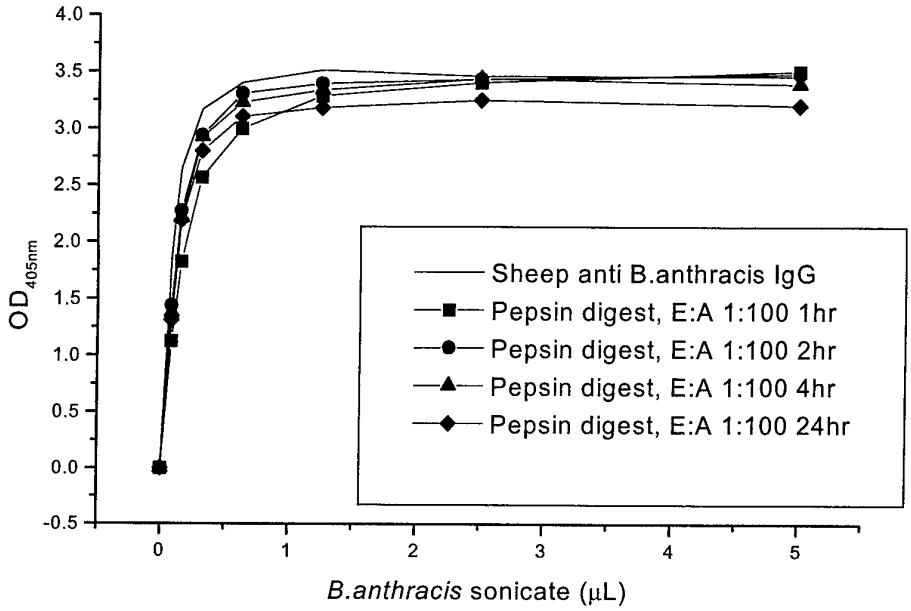


Figure 2. **Capture ELISA evaluation of antigen affinity of the intact and digested sheep anti-*Bacillus anthracis* polyclonal antibody.**

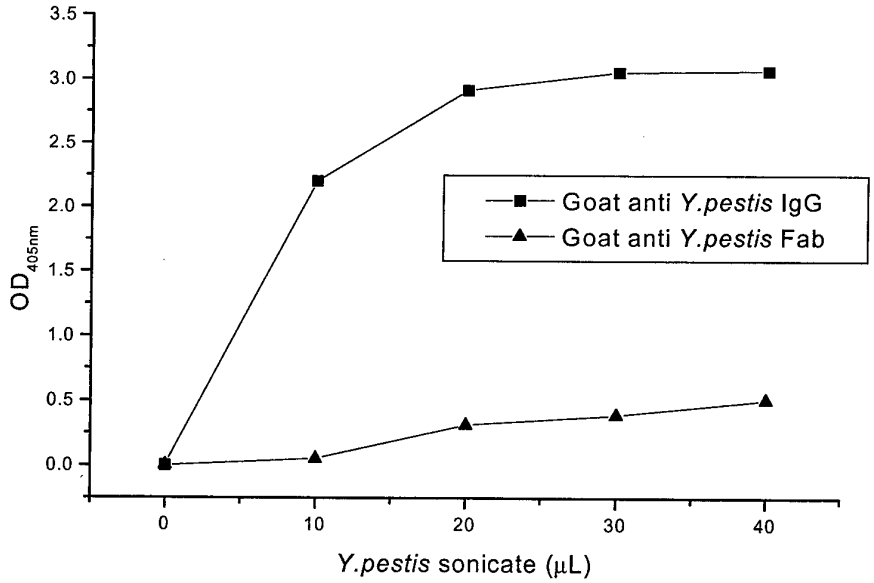
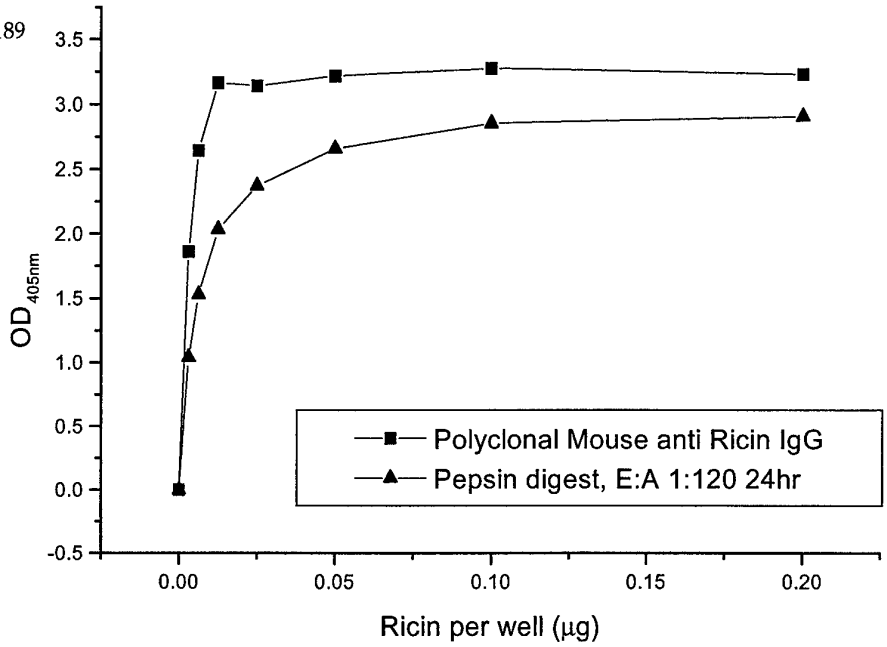


Figure 3. **Capture ELISA evaluation of antigen affinity of the intact and digested goat anti-*Yersinia pestis* polyclonal antibody.**



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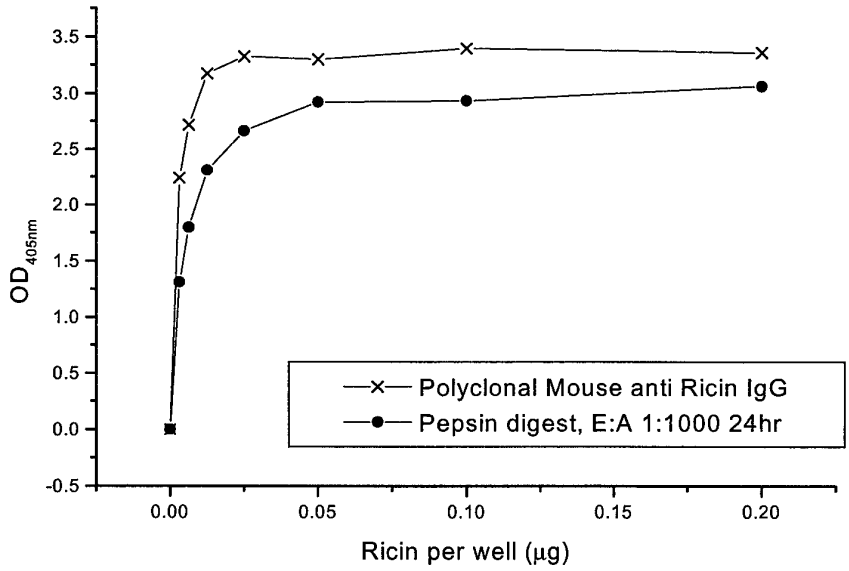

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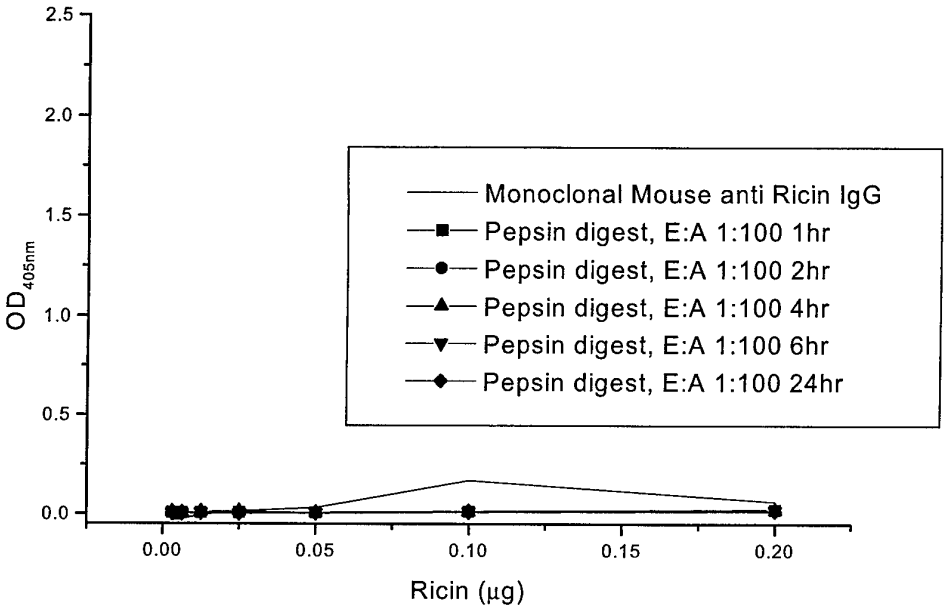
Figure 4. **Capture ELISA evaluation of antigen affinity of whole polyclonal mouse anti-ricin antibody and Fab produced using pepsin to antibody ratios of 1 to 120 (A) and 1 to 1000 (B).**



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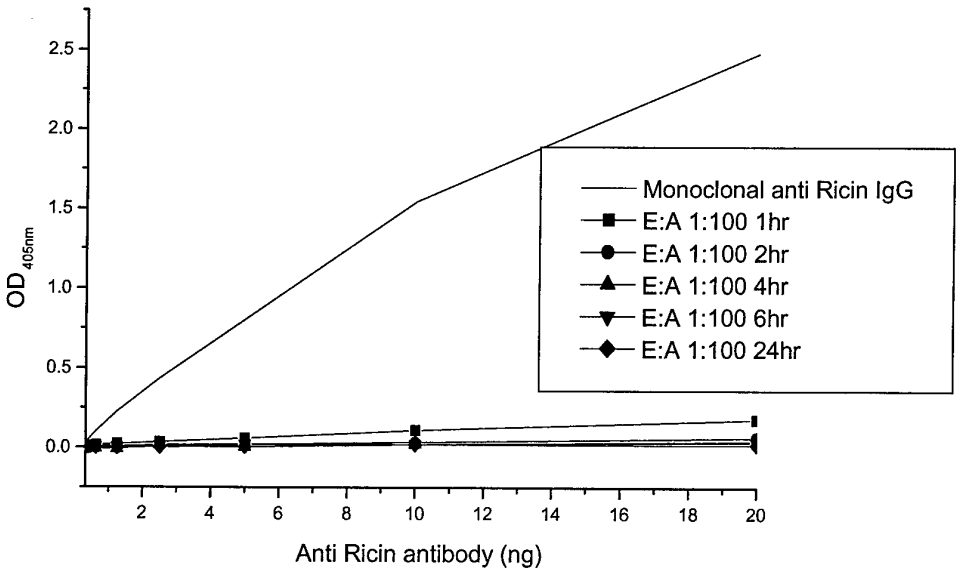

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Figure 5. **Comparison of antigen affinities for a mouse anti-ricin monoclonal antibody and F(ab′)$_2$ using a capture ELISA (A) and a direct ELISA (B)**

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A pepsin antibody fragmentation procedure was trialed and optimised. The procedure used was a modified version of that described in Current Protocols in Immunology. Pilot digestions using a range of pepsin concentrations and incubation times were completed at pH 4.0 and pH 4.5. The digestion products were examined by Phastgel electrophoresis, and the combination of pepsin concentration, incubation time and pH that provided the most efficient digestion of the antibody into dimeric antigen-binding fragments (F(ab')2) was then used in the large-scale digestion of the antibody. Monomeric antigen binding fragments (Fab) were obtained through reduction of F(ab')2 using 2-mercaptoethanol. In this study Fab from a mouse polyclonal anti-ricin antibody and a sheep polyclonal anti-Bacillus *anthracis* antibody generated with the optimised procedure were comparable to the whole molecule when used as the capture antibody in an ELISA. Nevertheless, it was noted that, in spite of meticulous optimisation of the digestion process it can be fraught with problems.

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