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Optimisation of the detection of bacterial proteases using adsorbed immunoglobulins as universal substrates

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ABSTRACT

Bacterial proteases, Type XXIV from *Bacillus licheniformens* and Type XIV from *Streptomyces griseus*, were used to investigate the utility and optimisation of a solid phase assay for proteases, using immunoglobulin proteins as substrates. Immunoglobulins IgA and IgG were adsorbed on to surfaces of ELISA plates and exposed to various levels of the bacterial proteases which led to digestion and desorption of proportional amounts of the immunoglobulins. The assay signal was developed by measuring the remaining proteins on the polystyrene surface with appropriate enzyme-labelled anti-immunoglobulin reagents. The assay was fully optimised in terms of substrate levels employing ELISA techniques to titrate levels of adsorbed substrates and protease analytes. The critical factor which influences assay sensitivity was found to be the substrate concentration, the levels of adsorbed immunoglobulins. The estimated detection limits for protease XXIV and XIV were 10 μ units/test and 9 μ units/test using IgA as a substrate. EC_{50} values were calculated as 213 and 48 μ units/test for each protease respectively. Using IgG as a substrate, the estimated detection limits were 104 μ units/test for protease XXIV and 9 μ units/test for protease XIV. EC_{50} values were calculated at 529 μ units/test and 28 μ units/test for protease XXIV and XIV respectively.

The solid phase protease assay required no modification of the substrates and the adsorption step is merely simple addition of immunoglobulins to ELISA plates. Adsorption of the immunoglobulins to polystyrene enabled straightforward separation of reaction mixtures prior to development of assay signal. The assay exploits the advantages of the technical facilities of ELISA technology and commercially available reagents enabling the detection and measurement of a wide range of proteases. However, the key issue was found to be that in order to achieve the potential performance of the simple assay, optimisation of the method was essential.

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1. Introduction

Study of the digestion of immunoglobulin proteins by proteases is important for several reasons including the need to understand neutralisation of antibody activity by microbial proteases and its impact on immunity [1], the controlled processing of immunoglobulins to investigate structure–function relationships [2], solubilisation of immune complexes [3], possible use in therapy of antibody-mediated diseases [4] and for the production of antibody fragments for use in immunochemical techniques and in therapy [5]. Defence antibodies, produced as part of the body's survival strategy against infectious agents, are assumed to be eliminated by secreted proteases from invading microbes as an element of facilitating bacterial colonisation. In this context the interest in the degradation of immunoglobulins by microbial proteases is focused on the study of proteases secreted by particular microorganisms and the specificity of the digestion process with reference to microbial virulence factors [6] and target body proteins such as members of the immunoglobulin class. Digestion of IgA by microbial enzymes has been of special interest due to the immunity functions of IgA as a secretory antibody to protect exposed surfaces from infectious microbes [7–13]. In addition to the well characterised digestion of IgA by microbial proteases, IgG class is also a target for microbial digestion [14,15]. Proteolysis of IgG has been shown to occur in several parts of the body including urinary and reproductive tracts [16]. In vitro controlled digestion of immunoglobulin proteins (mainly IgG) is a main stream possessing technique based on the use of specific enzyme, principally papain and pepsin, to generate F(ab′) and $F(ab')_2$ antibody fragments [17] for various purposes including therapeutic applications [18–20].

Despite the fact that the large number of studies on the digestion of immunoglobulins has demonstrated that this class of proteins

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function as particularly excellent macromolecular substrates for proteases, little attention has been given to using the principle for constructing assay method for the measurement of proteases in biological fluids or in other practically important applications. Measurement of protease activities in biological [21–24], industrial [25,26] and environmental samples [27,28] is an important section of bio-analysis.

Peptide-based [29–31] and several whole protein-based (other than immunoglobulins) substrates with various detectable labels have been described. Of particular interest is the universal type of substrates where whole labelled proteins were employed. Fluorescent and chromogenic labels as well as radioisotopes have been used to label casein, gelatin and albumin to prepare universal protease macromolecular substrates [32–40]. This type of substrates enabled the construction of relatively simple and useful methods for rapid measurements of proteases in many important application areas.

In this report, we demonstrate the use of IgG and IgA as solid phase macromolecular substrates for the measurement of proteases. The solid phase method is coupled with an ELISA end point detection step in order to produce an amplified detectable signal. The study demonstrates the use of human IgA and sheep IgG as substrate proteins with antigenic activities that are structure-dependent where degradation of the immunoglobulins results in loss of antigenic sites. The solid phase presentation of the substrates enabled the easy separation of digested fragments, which detach from the polystyrene surface, prior to the signal detection step. The results demonstrate a number of interesting technical features that may be applied to assaying proteases using immunoglobulins as substrates and provide an indication of how the new method may be developed and exploited in a wide range of application areas.

2. Experimental

2.1. Reagents and chemicals

All solvents, chemicals and buffer salts used in the present study are of analytical grade, were used without prior purification and obtained from BDH, Poole, Dorset, UK. Anti-sheep IgG (whole molecule)-Peroxidise antibody produced in donkey, Human IgA, Proteases Type XXIV from *Bacillus licheniformens* (10 units mg^{-1}) and Type XIV from *Streptomyces griseus* (35 units mg^{-1}), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma Chemical Company, Poole, Dorset, UK. Microtitre plates (96-well, flat bottom, high bind) were obtained from Greiner Bio-One Ltd., Stonehouse, Gloucestershire, UK. Water was purified with a Milli-Q 185 filtration plant from Millipore. Anti-human IgA (sheep) was obtained from MicroPharm, Wales. The antiserum was precipitated with 50% saturated ammonium sulphate and the precipitate was washed once with 45% saturated salt. Nutrient Agar was obtained from Oxoid, Basingstoke, Hampshire, UK and MacFarland standards were purchased from Pro-Lab Diagnostics, South Wirral, Cheshire, UK. Ultrasonic equipment was obtained from Sonics & Materials (UK) Ltd., Tomo Business Park, Stowmarket, Suffolk, UK and ELISA plate reader MRX II was obtained from Dynex Technologies Limited, Worthing, West Sussex, UK.

2.2. Buffers

(1) The buffer used for coating of the immunoglobulin substrates was 50 mM phosphate, pH 7.4, containing 100 mM NaCl.
(2) Protease dilution buffer (Tris buffer A) used was 20 mM Tris; 100 mM NaCl; 1 mM $CaCl_2$; 1 mM $ZnCl_2$; 1 mM $MgCl_2$, pH 7.4.
(3) Microtitre well blocking buffer was 20 mM Tris buffer pH 7.8 containing 100 mM NaCl; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 0.01% thimerosal and 0.05% Tween 20 as a preservative and 2% milk powder.
(4) Anti-immunoglobulin and anti-sheep HRP conjugate dilution buffer used was 50 mM phosphate buffer pH 7.4 containing 0.1 M NaCl, 0.2% gelatine and 0.01% thimerosal.
(5) Signal development buffer used was 50 mM acetate buffer, pH 4.1, containing H_2O_2.
(6) Microtitre well washing buffer used was 100 mM bicarbonate buffer containing 100 mM NaCl and 0.05% Tween 20.

2.3. Sheep IgG preparation

Immunoglobulin G was isolated from sheep serum according to previously published reports [41]. Briefly, pure immunoglobulins were isolated from the sheep antiserum by antigen-based affinity chromatography using cholic acid-Sepharose 4B immunosorbent gel. After dialysis the purified immunoglobulin samples were concentrated and passed through a DEAE-Sephadex A50 column (20 mm × 18 mm) to remove fines and unwanted traces of proteins.

2.4. Bacterial cultures

Colonies of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia* isolated from Nutrient Agar were inoculated into 5 mL autoclaved phosphate buffer (PBS). Suspension for each bacteria were prepared according to the reference of turbidity equivalent to the McFarland standard 0.5. This corresponded to 1.5×10^8 CFU mL^{-1} and referred to as the starter culture. Serial dilutions of 1/10 of the starter cultures were prepared in 20 of PBS in 50-mL Falcon tubes to provide the following microbial content: 1.5×10^8, 1.5×10^7, 1.5×10^6, 1.5×10^5 and 1.5×10^4 CFU mL^{-1}. The preparations (20 mL of each) were sonicated (according to manufacturer's instructions Sonics and Materials Inc.) and centrifuged to obtain supernatants ready for use in the protease assays.

2.5. Experimental design

In this study two classes of immunoglobulins, IgG and IgA, were used as solid phase substrates to develop simple and convenient assays for the detection and measurement of hydrolytic activity of proteases. Purified sheep IgG and human IgA were used in the development of solid phase assay methods which incorporated ELISA steps to develop the final signal. The immunoglobulins were prepared in stock solutions and the concentrations were estimated by measuring the absorbance at 280 nm and applying the equation: $E^{1\%}_{280\,nm} = 14.0$.

The immunoglobulin solutions were adjusted to working concentrations and serially diluted directly in the 96-well microtitre ELISA plates and allowed to adsorb. The adsorbed proteins were exposed to proteases under given conditions where hydrolysis of the adsorbed immunoglobulin resulted in loss of structural antigenic sites from the polystyrene surface. Hydrolysis of adsorbed immunoglobulins leads to desorption of the resulting fragments from the solid phase surface and decrease of remaining antigenic activity of the attached proteins. The assay signal was thus developed by measuring the remaining activities, using appropriate anti-immunoglobulin antibodies labelled with horse radish peroxidase, after washing to remove solubilised (detached) fragments and therefore the final signal level was inversely proportional to the concentration of added proteases. Essentially the assay is a tandem method in which the protease hydrolysis step is coupled to an ELISA for signal development. The solid phase protease assays differed according to the immunoglobulin used as the substrate and the manner by which the final signal was generated.

2.6. Assay steps

The protease assay with IgA and IgG as substrates was carried out in 96-well microtitre plates in which the immunoglobulin proteins were adsorbed to the polystyrene surface in the coating step. The immunoglobulins were diluted to the required concentration in phosphate buffer (50 mM Na_2HPO_4; pH 7.4, with 100 mM NaCl) and 100 μL volumes were transferred to the wells. Although the concentration values of the immunoglobulins in the coating buffer are known (and quoted in the study as such), the actual amounts of the attached protein (effective substrate concentrations) is not known. After coating for 16 h at 4 °C, the plates were washed 3 times with the plate washing buffer (50 mM sodium bicarbonate, 0.1 M NaCl, 0.05% Tween 20) and once with Tris buffer A (20 mM Tris; 100 mM NaCl; 1 mM $CaCl_2$; 1 mM $ZnCl_2$; 1 mM $MgCl_2$, pH 7.4). Protease preparations (100 μL) were diluted to the required concentration in the Tris buffer A and added to the plates. After incubation for 16 h at 37 °C, the plates were washed 4 times and left to stand for 50 min at room temperature with 250 μL of blocking buffer (20 mM Tris; 100 mM NaCl; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 0.01% thimerosal; 0.05% Tween 20 pH 7.8; with 2% milk powder).

After the blocking step, the plates were washed 4 times and the assay signal was developed with the second antibody-HRP method.

2.6.1. Assays using IgG as a protease substrate

When sheep IgG was used as antigenic protease substrate, after incubation with proteases and washing the plates, the assay signal was generated by adding 150 μL of anti-sheep HRP conjugate (diluted to 1/2000 in 50 mM sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl, 0.2% gelatine and 0.01% thimerosal) to all wells and the plates were incubated for 1 h at 37 °C. After washing the Plates 4 times, the HRP activity was measured by adding 150 μL of the ABTS substrate system (0.5 mg mL^{-1} ABTS in 50 mM sodium acetate/citrate buffer, pH 4.1, containing H_2O_2). The absorbance was read at 405 nm in a Dynex MRX II plate reader.

2.6.2. Assays using human IgA as substrate

When human IgA was used as antigenic protease substrate, after exposure to proteases and washing of assay plates, the assay signal was generated by a two-step procedure. Sheep anti-human IgA antibody (150 μL of 1/8000 in 50 mM sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl, 0.2% gelatine and 0.01% thimerosal) was added to all wells and the plates were incubated for 1 h at room temperature. After washing, 150 μL of anti-sheep HRP conjugate was added and the assay was completed as above.

2.7. Optimisation of assays

The measurement of proteases according to this protocol involved use of adsorbed immunoglobulin proteins, incubation with varied levels of proteases, incubation with signal developing reagents, and finally developing the colour with the HRP-labelled second antibody reagent.

In order to determine conditions to be used to develop assays with the maximum sensitivity, optimal assay conditions were determined in terms of the levels of the key reagents: the immunoglobulin substrate and the test enzymes. The optimisation steps were carried out by checkerboard titration tests in the 96-well plates.

IgG or IgA preparations were coated in serial dilutions starting at 33.3 μg mL^{-1} (all wells in column 1) and serially diluting to 0.0002 μg mL^{-1} (wells in column 12).

The activity levels in the commercial protease preparations used in subsequent assays were given by the suppliers as stated in the materials section. The values were used in the calculation of the activity (units mL^{-1}) used in the experiments (Section 2.8). Protease stocks were prepared by dissolving 10 mg of the protease (commercial powder preparations) in 10 mL of ice cold Tris buffer A. The protease preparations were stored as 1 mL aliquots at −20 °C until use.

After coating and washing, protease preparations (25,000 μ units/test for protease type XXIV and 8750 μ units/test for protease type XIV) was added to all wells in row A and serially diluted down the ELISA plate to reach 2 μ units/test and 0.5 μ units/test respectively for each protease in wells of row H. Following incubation, washing and blocking steps, the signal developing steps were carried out as described above according to nature of the immunoglobulin substrate (IgG or IgA). The assay signal was developed using the anti-sheep antibody-HRP in a fixed dilution 1/2000 added to all wells. After incubation the colour was developed as above.

The optimisation test enabled the selection of optimal conditions of the antibody that allow for the largest decrease in signal. The test was carried out using different antibody dilutions; various proteases and varied incubation conditions to further define optimal assay conditions.

2.8. Dose–response assays

IgG or IgA preparations were coated at concentrations of 0.6 μg mL^{-1} and 0.125 μg mL^{-1} respectively. These concentrations were selected from the defined optimal assay conditions carried out in the previous experiments. After coating and washing, protease preparations (Proteases Type XXIV from *B. licheniformens* and Type XIV from *S. griseus*) starting at 25,000 μ units/test for protease type XXIV and 8750 μ units/test for protease type XIV were added in triplicates and serially diluted to 2 μ units/test and 0.5 μ units/test respectively. Following incubation for 16 h at 37 °C and subsequent washing and blocking steps, the signal developing steps were carried out by adding anti-sheep antibody-HRP at a fixed dilution 1/2000 to all wells. After incubation for 1 h at 37 °C the colour was developed using the ABTS substrate system. EC_{50} values were calculated using a four parameter logistic curve.

2.9. Assessment of proteolytic activity by cultured bacteria

IgA and IgG were coated at fixed concentrations 0.125 μg mL^{-1} and 0.6 μg mL^{-1} respectively for 16 h at 4 °C. After coating, the plates were washed 3 times with the plate washing buffer. In order to determine the activity of the active proteases in bacterial culture supernatants, prepared as described in 2.4, were added in triplicates (100 μL) to coated wells and incubated for 16 h at 37 °C. Following incubation and subsequent washing and blocking steps, signal development steps were carried out as described above for IgG (Section 2.6.1) and IgA (Section 2.6.2).

3. Results and discussion

3.1. Optimisation of assays

In this study immunoglobulins were added to wells in coating solutions at specified concentrations that were estimated according to the accepted equation of absorbance at 280 nm. The monolayer of immunoglobulin molecules remains attached to the surface, unless digested by proteases, whereby the resulting smaller fragments detach into the solution and are washed off in subsequent steps prior to signal development. Undigested, and presumably the larger fragments of partially digested molecules, remain adsorbed and act as antigens in the signal development steps. Therefore, the assay signal is inversely proportional to the concentration of the proteases added and to the length of time allowed for digestion. The inverse relationship between the assay signal and

target protease activity mean that it is imperative that the level of adsorbed immunoglobulin (substrate) is carefully adjusted to enable maximum potential sensitivity to be achieved and a proportional response over a wide range of protease concentrations to be obtained. In the study two microbial protease preparations were employed in conjunction with ELISA technology to demonstrate an optimisation protocol to identify the critical parameters of the assay and to attain the potential maximum performance.

Results of the optimisation experiments with the two proteases (Type XXIV from *B. licheniformens* and Type XIV from *S. griseus*) showed that in the absence of proteases (control wells), as the concentration of adsorbed immunoglobulins increased, the signal (ELISA assay) increased across the whole titration range, as expected. This expected effect was due to the increasing concentrations of antigen sites to which the labelled antibody bound. The results are illustrated in Tables 1 and 2 (control rows, wells 1–12). However, in wells where proteases were added, as the levels of adsorbed immunoglobulins rose, the signal increased but to a significantly lower degree. This effect is seen when the absorbance values are viewed from left to right and compared with the control row. The degree of signal reduction in the test wells (rows A–H in Tables 1 and 2) was proportional to the concentration of proteases added to the respective rows. This effect is demonstrated by a gradual decrease in absorbance values as the protease concentrations increased, from 2 to 25,000 μ units/test for protease Type XXIV and 0.5–8750 μ units/test for Type XIV (equivalent to 4–25,000 ng mL^{-1}). The results of the optimisation assays demonstrate the critical effect of substrate concentration on the response profile of increasing protease levels. In Table 1A and B, the effect of adding proteases XXIV and XIV, is only seen when the IgA concentration (in the coating solution) is below 0.14 μg mL^{-1} (column 6 and beyond). It is also interesting to note that as the adsorbed IgA decreases; the absorbance profile in relation to protease concentrations soon reaches lower values than can be usefully employed for practical assays (columns 8–12). The tests indicate that there is an optimal "region" of substrate concentration where proportional response to the protease concentration range can be obtained. Under the test conditions described, this region is 0.05–0.14 μg mL^{-1} of IgA. Similar effects and hydrolytic activity are seen for both proteases with IgA as the substrate.

The pattern of response in the optimisation tests using sheep IgG is similar to the IgA results, but the optimal concentrations of IgG and the response profiles are quite different (Table 2A and B). Optimal IgG concentration region is between 0.02 μg mL^{-1} and 1.23 μg mL^{-1}. The hydrolytic activity of protease XXIV is much greater than that seen for protease XIV. This is easily seen when absorbance profiles in relation to protease concentrations are compared (columns 4–9). There is almost complete hydrolysis of the IgG with protease type XXIV and exposure to protease XIV did not lead to complete hydrolysis of the IgG which indicates a degree of specificity. More significantly, the hydrolytic activity profiles with IgG as the substrate are much greater (higher activity) than that obtained with IgA. This effect was unexpected due to commonly held understanding of IgA as a natural substrate for bacterial proteases [7–13] and limited information found in the literature reporting of protease digestion of IgG.

3.2. Dose–response graphs

A simple ELISA procedure was constructed for the determination of protease activity using adsorbed IgA and IgG using data obtained from the optimisation experiments as described in Section 2.8. Dose–response curves were constructed for each of the proteases using coated IgA at a concentration of 0.125 μg mL^{-1} and coated IgG at a concentration of 0.6 μg mL^{-1}. The range of protease concentration in the standard solutions was 4–25,000 ng mL^{-1} corresponding to the following ranges of protease units: 2–25,000 μ units/test

Table 1
Checkerboard titration tests of IgA with bacterial proteases. Varying concentrations (33.3–0.0002 μg mL^{-1}) of IgA were coated onto the microtitre plates. Varying concentrations of the two proteases Type XXIV from *Bacillus licheniformens* (2–25,000 μ units/test) and Type XIV from *Streptomyces griseus* (0.5–8750 μ units/test) were allowed to hydrolyse the IgA substrate. Signals were generated as described in Section 2. Absorbance values are shown.

(A)

XXIV	Protease (μ units/test)	1	2	3	4	5	6	7	8	9	10	11	12
		33.3[a]	11.1[a]	3.7[a]	1.23[a]	0.41[a]	0.14[a]	0.05[a]	0.02[a]	0.01[a]	0.002[a]	0.0006[a]	0.0002[a]
	Control	**3**	**3**	**3**	**3**	**3**	**3**	**1.8**	**0.78**	**0.43**	**0.24**	**0.23**	**0.21**
A	2	3	3	3	3	3	3	1.74	0.77	0.35	0.23	0.16	0.16
B	10	**3**	**3**	**3**	**3**	**3**	**3**	**1.68**	**0.75**	**0.34**	**0.23**	**0.16**	**0.15**
C	20	3	3	3	3	3	3	1.66	0.72	0.33	0.21	0.16	0.15
D	100	**3**	**3**	**3**	**3**	**3**	**2.69**	**1.32**	**0.54**	**0.28**	**0.17**	**0.15**	**0.13**
E	400	3	3	3	3	3	2.29	1.08	0.47	0.26	0.16	0.14	0.13
F	1600	3	3	3	3	3	1.99	0.86	0.40	0.22	0.15	0.13	0.13
G	6300	3	3	3	3	3	1.34	0.54	0.25	0.18	0.14	0.12	0.12
H	25,000	3	3	3	3	3	0.79	0.37	0.20	0.14	0.13	0.11	0.11

(B)

XIV	Protease (μ units/test)	1	2	3	4	5	6	7	8	9	10	11	12
		33.3[a]	11.1[a]	3.7[a]	1.23[a]	0.41[a]	0.14[a]	0.05[a]	0.02[a]	0.01[a]	0.002[a]	0.0006[a]	0.0002[a]
	Control	**3**	**3**	**3**	**3**	**3**	**3**	**1.8**	**0.99**	**0.43**	**0.26**	**0.23**	**0.21**
A	0.5	3	3	3	3	3	3	1.58	0.78	0.43	0.24	0.21	0.21
B	2	3	3	3	3	3	2.91	1.57	0.78	0.41	0.24	0.20	0.19
C	9	**3**	**3**	**3**	**3**	**3**	**2.90**	**1.53**	**0.77**	**0.40**	**0.22**	**0.19**	**0.17**
D	34	3	3	3	3	3	2.89	1.43	0.71	0.40	0.22	0.19	0.17
E	137	**3**	**3**	**3**	**3**	**3**	**2.61**	**1.34**	**0.58**	**0.33**	**0.18**	**0.17**	**0.16**
F	547	3	3	3	3	3	2.26	1.02	0.49	0.27	0.18	0.16	0.16
G	2188	3	3	3	3	3	2.05	0.95	0.45	0.24	0.17	0.15	0.14
H	8750	3	3	3	3	3	1.65	0.69	0.33	0.21	0.15	0.13	0.13

The highlighted values in the tables are for comparison purposes to highlight the key differences in the action of the two proteases. The bold values in the tables are for demonstrating the varied protease action on the IgA substrate and the IgG substrate.

[a] IgA (μg mL^{-1}).

Table 2
Checkerboard titration tests of IgG with bacterial proteases. Varying concentrations (33.3–0.0002 μg mL^{-1}) of IgG were coated onto the microtitre plates. Varying concentrations of the two proteases Type XXIV from *Bacillus licheniformens* (2–25,000 μ units/test) and Type XIV from *Streptomyces griseus* (0.5–8750 μ units/test) were allowed to hydrolyse the IgG substrate. Signals were generated as described in Section 2. Absorbance values are shown.

(A)

XXIV	Protease (μ units/test)	1	2	3	4	5	6	7	8	9	10	11	12
		33.3[a]	11.1[a]	3.7[a]	1.23[a]	0.41[a]	0.14[a]	0.05[a]	0.02[a]	0.01[a]	0.002[a]	0.0006[a]	0.0002[a]
	Control	**3**	**3**	**3**	**1.61**	**1.61**	**1.30**	**1.30**	**1.12**	**0.86**	**0.55**	**0.53**	**0.51**
A	2	3	3	3	0.39	0.22	0.23	0.21	0.18	0.17	0.17	0.17	0.16
B	10	**3**	**3**	**3**	**0.28**	**0.21**	**0.20**	**0.17**	**0.17**	**0.15**	**0.15**	**0.14**	**0.14**
C	20	3	3	3	0.27	0.17	0.15	0.15	0.15	0.13	0.13	0.13	0.13
D	100	**3**	**3**	**3**	**0.23**	**0.14**	**0.14**	**0.14**	**0.13**	**0.13**	**0.13**	**0.13**	**0.12**
E	400	3	3	3	0.20	0.14	0.13	0.13	0.12	0.12	0.12	0.12	0.11
F	1600	3	3	2.13	0.15	0.14	0.13	0.12	0.12	0.12	0.11	0.11	0.11
G	6300	3	3	1.08	0.14	0.13	0.13	0.12	0.11	0.11	0.11	0.11	0.11
H	25,000	3	3	0.67	0.14	0.12	0.12	0.11	0.11	0.11	0.11	0.11	0.10

(B)

XIV	Protease (μ units/test)	1	2	3	4	5	6	7	8	9	10	11	12
		33.3[a]	11.1[a]	3.7[a]	1.23[a]	0.41[a]	0.14[a]	0.05[a]	0.02[a]	0.01[a]	0.002[a]	0.0006[a]	0.0002[a]
	Control	**3**	**3**	**2.88**	**1.61**	**1.61**	**1.30**	**1.12**	**1.30**	**0.86**	**0.55**	**0.53**	**0.51**
A	0.5	3	3	2.55	1.50	1.46	1.13	1.00	0.94	0.65	0.38	0.36	0.33
B	2	3	3	2.45	1.30	1.15	1.08	0.93	0.90	0.61	0.42	0.34	0.30
C	9	**3**	**3**	**2.20**	**1.24**	**1.15**	**1.01**	**0.84**	**0.82**	**0.56**	**0.38**	**0.33**	**0.28**
D	34	3	3	2.11	1.04	0.80	0.80	0.78	0.78	0.42	0.32	0.28	0.24
E	137	**3**	**3**	**1.03**	**0.68**	**0.65**	**0.62**	**0.59**	**0.58**	**0.34**	**0.26**	**0.25**	**0.22**
F	547	3	3	0.61	0.44	0.42	0.39	0.39	0.36	0.24	0.23	0.20	0.18
G	2188	3	2.99	0.28	0.28	0.27	0.26	0.24	0.26	0.23	0.20	0.19	0.17
H	8750	3	1.34	0.23	0.20	0.18	0.18	0.18	0.18	0.18	0.18	0.16	0.16

The highlighted values in the tables are for comparison purposes to highlight the key differences in the action of the two proteases. The bold values in the tables are for demonstrating the varied protease action on the IgA substrate and the IgG substrate.

[a] IgA (μg mL^{-1}).

for protease XXIV and 0.5–8750 μ units/test for protease XIV. The dose–response curves with different proteases and different immunoglobulins are given in Figs. 1–4.

The curves shown in Figs. 1 and 2 show plots for each protease using adsorbed IgA. The estimated detection limits being 10 μ units/test for protease XXIV and 9 μ units/test for protease XIV. EC_{50} values were calculated as 213 μ units/test and 48 μ units/test respectively, using the same data analysis. The curves in Figs. 3 and 4 show plots for the each protease using adsorbed IgG with the estimated detection limits being 104 μ units/test for protease XXIV and 9 μ units/test for protease XIV. EC_{50} values were calculated at 529 μ units/test and 28 μ units/test respectively.

The described protease assay method is based on natural whole protein substrates presented in solid phase format. The use of immunoglobulins as universal protease substrates is intuitively driven because of the available vast information on bacterial inactivation of defence antibodies [7–13] and other sources where

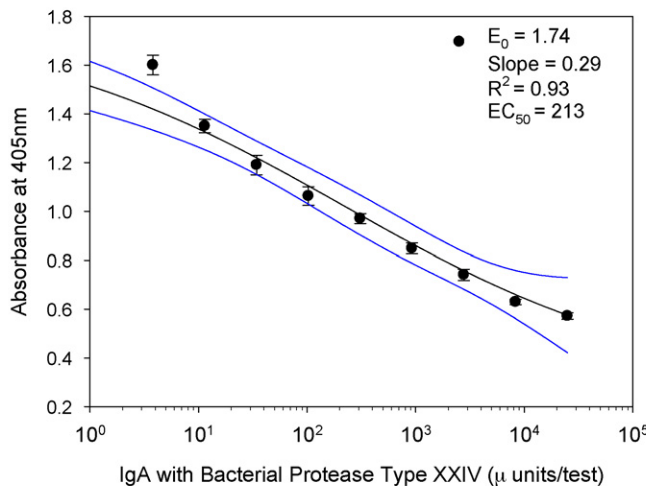

Fig. 1. Dose–response curve of bacterial protease Type XXIV from *Bacillus licheniformens* (specific activity 10 units/mg). Varying concentrations of protease were added to IgA (0.125 μg mL^{-1}) and allowed to incubate at 37 °C for 16 h. The signals were generated as described in Section 2.8. The values plotted are mean ± SD of three determinations.

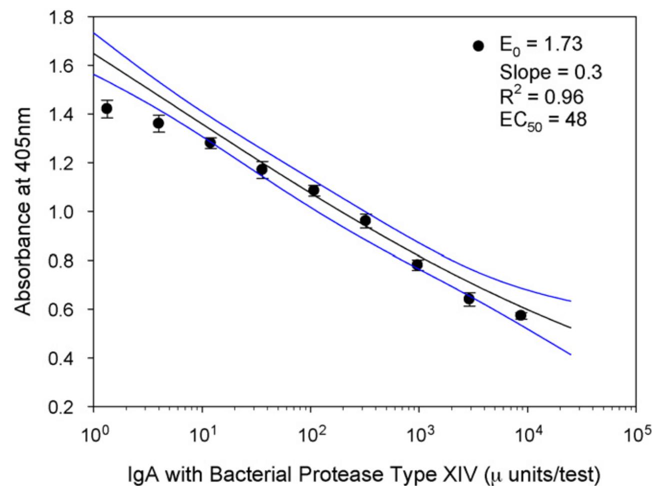

Fig. 2. Dose–response curve of bacterial protease Type XIV from *Streptomyces griseus* (S.A. 3.5 units/mg). Varying concentrations of protease were added to IgA (0.125 μg mL^{-1}) and allowed to incubate at 37 °C for 16 h. The signals were generated as described in Section 2.8. The values plotted are mean ± SD of three determinations.

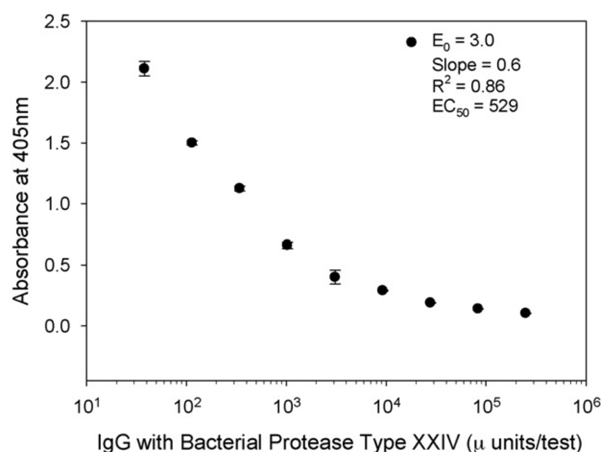

Fig. 3. Dose–response curve of bacterial protease Type XXIV from *Bacillus licheniformens* (S.A. 10 units/mg). Varying concentrations of protease were added to IgG (0.6 μg mL^{-1}) and allowed to incubate at 37 °C for 16 h. The signals were generated as described in Section 2.8. The values plotted are mean ± SD of three determinations.

immunoglobulins are digested in vitro for a purpose [17–20]. A recent report suggests the use of immunoglobulins as specific substrates for particular proteases [42]. Although this is probably a feasible approach, it is difficult to see how proteases in mixtures can be distinguished considering the broad susceptibility of immunoglobulins to a wide range of proteases. The solid phase presentation of unmodified immunoglobulins as universal protease substrates offers an interesting alternative to the commonly used proteins such as casein and albumin and exploits the wide availability of anti-antibody-enzyme reagents. Our findings clearly indicate that IgG and IgA can be used as generic substrates for the detection of proteolytic activity in samples. Although there were observed differences in the response of the proteases tested (and different bacterial species, Section 3.3) the immunoglobulins may be used as generic substrates for detection of total proteolytic activity according to the principle of using whole protein substrates [36,37]. The assay protocol described in this report exploits the generally understood ELISA technology and widely available instrumentation together with the advantages offered by the 96-well plates. The exceptionally easy steps of adsorption of IgA and IgG obviate the need for insolubilisation of protein substrates and provides a near perfect means of separating reaction products prior to signal development steps. The passive adsorption of IgA and IgG to polystyrene provides strong attachment to the surface but it is not completely irreversible. The development of the signal by measuring the remaining attached antigen (the unhydrolysed substrate) in the described protocol has a number of important advantages. It is technically and practically convenient, exceptionally cost-effective, it overcomes any interference that may result from desorption of substrate during the assay steps and it facilitates exploitation of the full advantages of ELISA technology including signal amplification and commercial availability of reagents.

The optimum levels of adsorbed IgG and IgA (the substrate concentration) were found to be in the low ng/well range. The estimated capacity of the surface areas of the wells (Greiner Bio-One 96-well, flat bottom, high bind) using 100 μL volumes is 0.92 cm^2. The estimated amounts of IgG and IgA providing monolayer coverage are 350 ng and 400 ng for IgG and IgA respectively [43]. The concentration of IgG used to coat the plates was 600 ng mL^{-1} and therefore it is reasonable to assume full coverage of the available surface. However, the concentration of IgA used for coating (125 ng mL^{-1}) would not be expected to provide a full coverage. Assuming 70% adsorption [44] the expected levels of IgA adsorbed to individual wells is approximately 88 ng. It is relevant to note that the amounts of immunoglobulin substrates used in the described assays and in previously reported methods [42] are far below the levels normally employed for protease assays [36,37]. This is defined by the working principle of the coated protein and signal determination protocol. It is plausible that in the absence of maximum coverage of the available surface area, the added proteases may adsorb to the unoccupied sites and become unavailable for proteolytic activity. However, in the present assays, the protease preparations were crude extracts (low specific activities: Type XXIV 10 units mg^{-1} and Type XIV 35 units mg^{-1}) containing high levels of non-enzyme proteins and therefore the proportion of pure enzyme molecules is negligible and therefore the loss of activity due to adsorption of enzymes is insignificant.

3.3. Response by cultured bacteria using adsorbed IgG and IgA

The four sonicated bacterial preparations of the four species tested (*E. coli*, *P. aeruginosa*, *S. aureus*, and *K. pneumonia*) gave varied levels of hydrolysis of the adsorbed immunoglobulins. The level of hydrolysis of adsorbed IgG was highest with *P. aeruginosa*, followed by *Klebsiella*, *Staphylococcus* and least degree of hydrolysis was shown by *E. Coli* (Fig. 5). The extent of hydrolysis is shown by the decrease in the measurable signal, the ELISA absorbance values. The signal from the control points was constant throughout, and in accordance with the assay principle, was the highest absorbance. The relationship between the bacterial content and signal was inversely proportional. The sensitivity and dynamic range was most pronounced in the *Pseudomonas* preparations suggesting that this bacterial species exhibits highest levels of proteolytic activity. This is in agreement with recently published data [45]. The dose–response relationship with *Klebsiella*, *Staphylococcus* and *E.coli* was significantly less pronounced suggesting the presence of lower levels of solubilised proteases.

The response observed with *Pseudomonas* preparation using adsorbed IgA as substrate was similar to that found with IgG, a high degree of hydrolysis and a pronounced dose–response relationship. However, as can be seen in Fig. 5B and 6B, the degrees of hydrolysis of the two immunoglobulins were significantly different with the proteolytic activity of IgG (0.6) as a substrate being almost double the response obtained with IgA (0.3) as a substrate when comparing the absorbance units obtained for the difference between the control samples and the highest microbial content

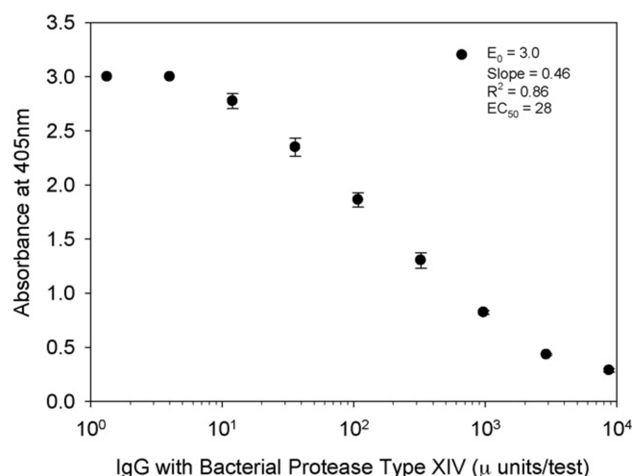

Fig. 4. Dose–response curve of bacterial protease Type XIV from *Streptomyces griseus* (S.A. 3.5 units/mg). Varying concentrations of protease were added to IgG (0.6 μg mL^{-1}) and allowed to incubate at 37 °C for 16 h. The signals were generated as described in Section 2.8. The values plotted are mean ± SD of three determinations.

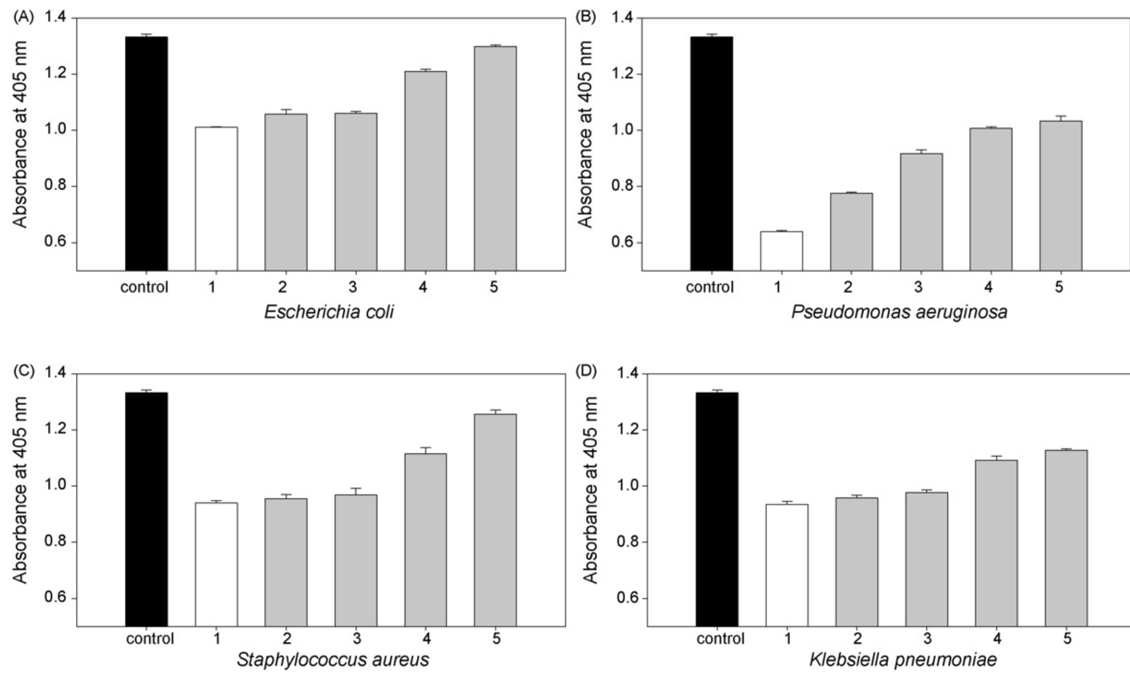

Fig. 5. Protease activities found in sonicated bacterial samples. The assays were conducted using adsorbed IgG as a substrate (0.6 μg mL^{-1}).

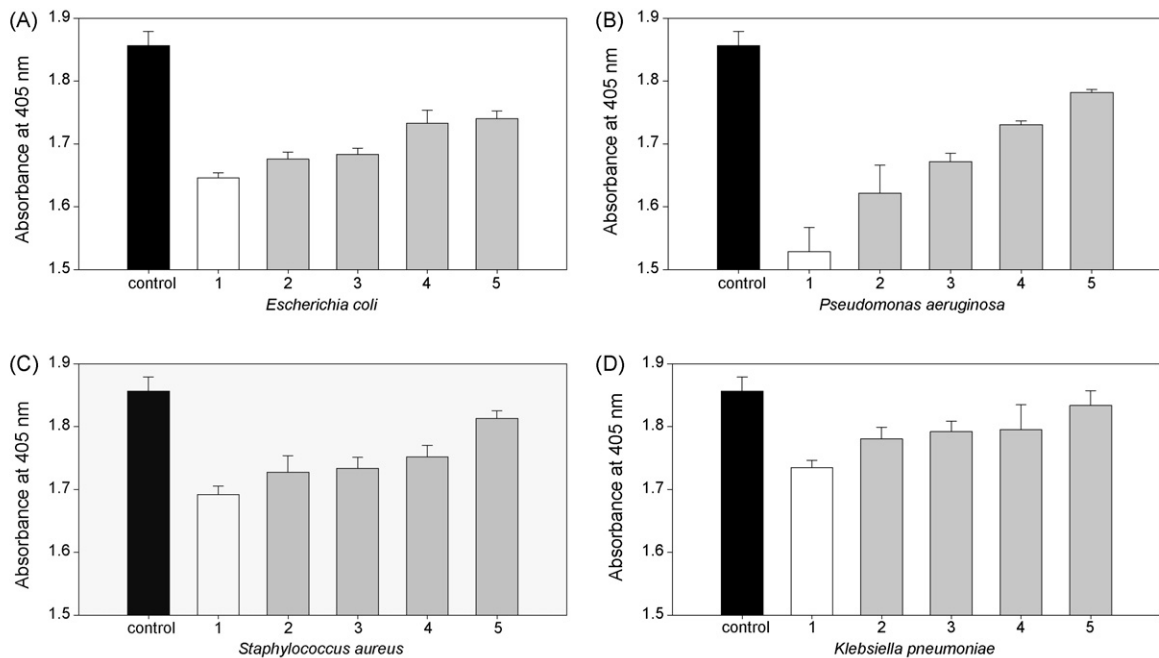

Fig. 6. Protease activities found in sonicated bacterial samples. The assays were conducted using adsorbed IgA as a substrate (0.125 μg mL^{-1}).

preparation. The hydrolytic activity response profiles obtained with the other microbes (Fig. 6) followed the order *E. coli* (highest), *Staphylococcus* and *Klebsiella* showing the lowest level of protease activity.

The response observed by the bacterial species tested indicates that the use of adsorbed immunoglobulins as generic protease substrates can be coupled with the convenience of the solid phase assay and the ELISA signal development protocol. It is interesting to note that the results using the sonicated bacterial preparations were in line with the results obtained using the commercial protease preparations (Section 2.8). Both groups of tests demonstrate higher activity (response) with adsorbed IgG compared with IgA. As noted earlier this finding was unexpected in view of the generally accepted view that IgA is the target substrate for microbial proteases during infection [1,5,6,9,10,17].

It is also relevant to point out that it may not be necessary to sonicate bacterial suspensions prior to assay for soluble (secreted) microbial proteases. Similar response profiles were obtained using whole cell preparations (data not shown). The tests indicated that it is entirely feasible to detect presence of viable bacteria by assaying activity of extracellular secreted biomarker proteases by the described method.

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