ELSEVIER

The Chemical Engineering Journal 65 (1997) 87–91

CHEMICAL ENGINEERING JOURNAL

Short communication

An investigation into the possible effects of proteolysis on IgM enzyme-linked immunosorbent assay titres

Papagudi Sriram, Nicolas Kalogerakis *, Leo A. Behie

Pharmaceutical Production Research Facility (PPRF), Faculty of Engineering, The University of Calgary, Calgary, Alta., T2N 1N4, Canada

Received 12 October 1995; accepted 12 March 1996

Abstract

On the basis of the theory of affinity it is shown that enzyme-linked immunosorbent assay titres cannot increase as a result of proteolysis when all the antibody proteolytic fragments have approximately the same association constants as the intact antibody for the reaction with the antigen and with the enzyme-conjugated antibody. This finding was experimentally confirmed by proteolysis experiments carried out with a monoclonal IgM antibody against Lewis^b antigen using trypsin. The experimental results with this particular antibody confirmed that proteolysis cannot enhance ELISA titres in hybridoma cultures with serum-free medium or with serum-supplemented medium.

Keywords: Proteolysis; ELISA titres; Antibody; Enzyme

1. Introduction

Monoclonal antibodies are widely used in diagnostic and therapeutics as a result of their antigenic determinant specificity [1–3]. Monoclonal antibodies are typically produced in vitro by hybridoma cells cultivated in tissue culture flasks or bioreactors. Occasionally, monoclonal antibodies are also produced in vivo in ascites fluid of a mouse.

The proteolysis of immunoglobulin by proteases results in the break-up of antibody molecules into smaller fragments. The proteolysis yields either "Fc" fragments which have lost the antigen-binding capabilities of the immunoglobulin or "Fab" assay (ELISA) is employed for the quantification of monoclonal antibody produced in hybridoma cell cultures. The ELISA assay uses the specific antigen-binding capabilities of the immunoglobulin to be assayed. Hence, the ELISA assay cannot cannot distinguish between an intact immunoglobulin molecule and its Fab fragments. In many applications of monoclonal antibodies, only the intact antibody molecules are useful, e.g. in haemagglutination assays where the ability of an IgM molecule to bind to more than one antigenic determinant site is important [6]. In the case of IgM production, proteolysis becomes an important issue [1]. Recently, the presence of serine proteases, which are neutral protease (active in pH range 6.0–9.0), has been reported in the literature for a hybridoma cell culture supernatant from serum-free media [7]. Acidic proteases (active in pH range 2.0–6.0), which are cathepsin D like proteases, has been reported by many workers for different hybridoma cell lines [8–11]. The proteolytic fragments encountered in the supernatant of serum-free hybridoma cultures have been reported by van Erp et al. [10].

In any ELISA assay, both the intact antibody and its Fab fragments are capable of being captured by the antigenic determinant site of the antigen used in the ELISA assay. Hence, the antibody titre obtained by ELISA for a proteolysed antibody sample is the cumulative effect of both the intact antibody to be assayed and its Fab fragments. Obviously, this may result in increased antibody titres. This possibility was considered as one of the probable explanations for the unusually high antibody titres obtained during the end of batch cultures [12]. A similar possibility was considered by Martens et al. [13] to explain the higher antibody yields at low dilution rates in their studies on chemostat hybridoma cultures. The underlying assumption behind these explanations is that the ELISA titres for the proteolysed sample of the intact immunoglobulin must be higher than those for the sample with intact immunoglobulin molecules.

In this paper, on the basis of the theory of affinity we arrive at qualitative conclusions regarding the effect of the presence of proteolytic fragments on the ELISA titres. A series of experiments were also carried out to study the effect of proteolysis by trypsin on ELISA titres of an IgM sample in

*Corresponding author. Present address: Bioengineering Laboratory, Chemical Engineering Department, State University of New York, Buffalo, NY 14260-4200, USA.

PII S1385-8947(96)03115-4

serum-free Dulbecco's modified Eagle's medium (DMEM) and in DMEM medium supplemented with 5% (by volume) foetal bovine serum (FBS).

2. Indirect method of enzyme-linked immunosorbent assay

This method is widely used for quantifying immunoglobulins in hybridoma cell cultures. In the first step, wells of the polystyrene microwell plates are sensitized by passive adsorption with the relevant antigen by incubating with antigen coating solution for a sufficient period of time. In some ELISA protocols there is a separate blocking step, where the remaining free adsorption sites on the plates are all blocked by passive adsorption of bovine serum albumin (BSA) on the plates. The second step is to incubate the test sample in the sensitized plate for a fixed period of time. The antibody present in the test sample reacts with the immobilized antigen on the well surfaces. The third step is to incubate with the enzyme-linked antibody conjugate (usually an IgG or Fab fragment of IgG) for a fixed period of time. This reacts with any antibody "captured" in the second step. The fourth step is to add enzyme substrate to these wells in the plate and incubate for some fixed time. Between each of the previously described steps, the plates are carefully washed. The rate of degradation of the substrate is indicated by the colour change, which is proportional to the antibody concentration in the test samples. The reaction is stopped and the colour change is quantitatively assessed in a spectrophotometer.

3. Theoretical results based on the theory of antibody affinity

On the basis of the theory of antibody affinity described by Day [4], the amount ξ (mol) of antigenic determinants bound to the antibody by the reaction in step 1 of an ELISA assay when the sample in a well of the ELISA microplate is an amount A (mol) of intact antibody per well was found to be related to the antibody concentration by a non-linear function [14]. When the concentrations of antigen and the antibody in the well are low and the binomial approximation is used, a simple linear relationship can be obtained for the reaction between the antigen and the sample antibody:

$$\xi = \frac{K_{12} s P}{1 + K_{12} s P} nA \tag{1}$$

Similarly, it can be shown [14] that the amount E (mol) of enzyme bound to the antigen–antibody complex in the well from the previous step can also be approximated by a linear relation with respect to ξ, i.e.

$$E = \frac{K_{ge} n_e C}{1 + K_{ge} n_e C} \gamma g \xi \tag{2}$$

Eq. (2) gives the number of moles of enzyme attached to the microwell surface after the washing step is carried out. If the amount of enzyme attached to the well surface, the substrate concentration S, and the time duration t of incubation with substrate are known, we can derive the expression for the measured difference in absorbance in ELISA on the basis of the linear relationship between the absorbance reading and the coloured protein present in a well as

$$\Delta I = \phi X = \phi V k_m E \int_0^t \frac{S}{K_m + S} \, dt \tag{3}$$

In ELISA, the samples are always diluted so as to be in the range where the standard antibody samples give linear variation in absorbance with antibody concentration. For a particular system and ELISA protocol the following quantities are constant: P, C, n_e, s, γ, ϕ, k_m, K_m, t, the valences of antibody and its fragments, and the association constants for all the antigen–antibody reactions. Typical values of various variables for the ELISA protocol used are given in Table 1. The above linear relationship between antibody concentration and absorbance reading can only be obtained when the antigen and antibody concentrations in the well are low.

Let us now consider an ELISA well plate sensitized with the same antigen; however, assume that the antibody sample has undergone proteolysis. The corresponding linear relationship for the reaction between the sample containing the proteolytic fragments of an amount A (mol) of intact antibody can be readily obtained for cases where the association constants for the intact antibody and its proteolytic fragments for the reaction with antigen as well as the enzyme conjugated antibody are not significantly altered by proteolysis. The corresponding equations for the amount ξ_m (mol) of antigenic determinants bound to the antibody and the total amount E_m (mol) of enzyme present in well are given by

$$\xi_m = \frac{K_{12} s P}{1 + K_{12} s P} \sum_{i=1}^{N} n_i A_i \tag{4}$$

and

$$E_m = \frac{K_{ge} n_e C}{1 + K_{ge} n_e C} \gamma \sum_{i=1}^{N} g_i \xi_i \tag{5}$$

Since proteolysis can only reduce the number of antigen-binding sites of molecules and the antigenic sites in heavy and light chains present in the attached antibody in the well, we have

$$\sum_{i=1}^{N} n_i A_i \leqslant nA \quad \text{and} \quad \sum_{i=1}^{N} g_i \xi_i \leqslant g\xi \tag{6}$$

Hence, if the association constants for the antigen–antibody reaction as well as the association constant for enzyme-conjugated IgG and antibody's heavy and light chain are approximately the same for antibody and its fragments, on the basis of Eqs. (1)–(6) it can be inferred that the ELISA titre will either remain the same or decrease after the sample has been

Table 1
Typical values of various parameters obtained from literature and the assay protocol for the enzyme-linked immunosorbent assay used in this study

Parameters	Units	Typical values
Amount of antigen Lewis [b] used for coating each well	$\mu g\ well^{-1}$	0.05
Molecular weight of Lewis blood group substances	Da	270 100 [a]
Amount of IgM used as standard in ELISA	ng $well^{-1}$ mol $well^{-1}$	0–40 $(0–0.44)\times10^{-14}$
Molecular weight of IgM molecule	Da	900 000
Valence of IgM	–	10
Valence of IgG	–	2
Association constants range [b]	l mol^{-1}	1×10^{4}–5×10^{7}
Valence of antigen reported in literature	–	1–2

[a] Data taken from Sober [16].
[b] Data from Day [4].

subjected to proteolysis. These results have been obtained by simple algebraic manipulations of the experimentally verified relationships used by immunochemists for the determination of the values of association constants for antigen–antibody interactions. Detailed derivations are given elsewhere [14].

As far as the assumption of approximately equal association constants, we have found in the literature reports where both IgM and IgG antibodies have similar association constants for the intact MAb molecule and its proteolytic fragments [15,16]. There is also experimental evidence reported in the literature for rat and mouse antibodies indicating that the association constant for such antigenic interactions of antibodies and its antiserum is not significantly altered by proteolysis [5].

4. Materials and methods

The murine IgM antibody against Lewis[b] carbohydrate structures used for the experiments was obtained from Chembiomed Ltd. (Edmonton, Alberta) as a solution of 1 g IgM l^{-1} in 1.0% BSA. The trypsin from bovine pancreas used for proteolysis was obtained from Sigma Chemical Co. (Lot No. 31H0420). The soyabean trypsin inhibitor was obtained from Boehringer Mannheim (Lot No. 12890021-37). The phosphate-buffered saline (PBS) used in these experiments was prepared using powder obtained from Sigma Chemical Co. (Catalogue No. 1000-3) which contained 120 mM NaCl, 2.7 mM KCl and 10 mM of phosphate buffer salts with pH adjusted to 7.4. The FBS was obtained from Gibco (Lot No. 42N7125). Affinity-purified synthetic antigen Lewis[b] obtained from Chembiomed. Ltd. was used for sensitizing the ELISA plates.

4.1. Enzyme-linked immunosorbent

The wells of the 96-well microplate (Nunc) were first sensitized by incubating overnight with 100 μl of 5 mg synthetic Lewis[b] antigen l^{-1} in PBS at room temperature. During this period the passive adsorption of antigen molecules on the well surface takes place. The plates are then washed with wash buffer solution containing 1.0% Tween 20 and 1.0×10^{-4} mol Merthiolate (Thimerosal) l^{-1} in PBS. The 100 μl of the ELISA samples diluted in dilution buffer (1.0% Tween 20, 1.0% BSA and 1.0×10^{-4} mol Merthiolate) l^{-1} was loaded together with the dilution buffer as blanks and the standard antibody solution in PBS in the concentration range 0–40 mg l^{-1}. The wells were covered and incubated at 37 °C for 1 h. During this time the unoccupied adsorption sites were blocked by adsorption of BSA and the antibodies in the sample attached themselves to the adsorbed antigen molecules. The plates were then washed with wash buffer. Subsequently, 100 μl of horseradish peroxidase conjugated antibody (goat anti-mouse IgG [H+L] horseradish peroxidase conjugate, Bio-Rad Laboratories Catalogue No. 172-1011) solution in dilution buffer containing 10 mg l^{-1} of enzyme-conjugated antibody was loaded in each well and the plate was again covered and incubated for 1 h at 37 °C. The plates were washed and the 100 μl of substrate buffer containing 3.0 g l^{-1} of *o*-phenylene diamine and 668 μl of 36% hydrogen peroxide solution in 1 ml of citric acid buffer (4.67 g citric acid l^{-1}, 7.9 g Na_2HPO_4 l^{-1}, pH 5.5) was loaded in each well. The plates were incubated for about 5 min and the reaction was stopped by adding 100 μl of 1 N hydrochloric acid solution to each well and the absorbance of 405 nm wavelength light in each well was automatically determined using an EL 340 optical plate reader (Bio-Tek Instruments). The calibration curve was determined using the standard solution absorbance by linear regression. The final concentration was the average of three different measurements at two dilutions (total of six measurements per sample).

4.2. Proteolysis experiments

In these experiments, the objective was to study the variations in ELISA titres obtained for the IgM antibody sample

after it had undergone proteolysis for different periods of time. The interest was mainly in determining whether there is an increase in ELISA titres after proteolysis. This would indicate that higher ELISA titres obtained during the end of batch cultures can indeed be due to proteolysis of the antibody molecule. Two reaction mixtures were prepared: one containing 4 mg l^{-1} of trypsin in PBS and the other containing 4 mg l^{-1} of trypsin in PBS containing 5.0% (by volume) FBS. To each one these mixtures the purified IgM was added and the total volumes of the reaction mixtures were 8.0 ml each. The reaction mixtures were incubated at 37 °C. From each of the two reaction mixtures 1.0 ml of sample was taken at regular intervals and poured into 1.0 ml of the 1.0 g soyabean trypsin inhibitor l^{-1} solution in PBS to arrest the proteolysis reaction and stored at −20 °C prior to analysis. Then ELISA was carried out for each of these samples. The concentration of trypsin was chosen to be 4 mg l^{-1}, since experimental results on the autolysis of trypsin have shown that even after eight days there is significant trypsin activity present and the trypsin activity is not completely lost [15].

Results and discussion

The results of ELISA of the samples of the proteolysis experiments are shown in Fig. 1. As seen, the ELISA titres never increased from their initial value for both the reaction mixtures even after the treatment with trypsin has proceeded for a considerable period of time. When no FBS is present, the ELISA titres remained nearly constant for the samples with up to 42 h of treatment with trypsin and then they decreased by about 20% for the sample obtained after 118 h of treatment with trypsin. When pure samples of IgM are treated with trypsin at a pH value of 8.0 and an enzyme-to-substrate concentration of 1:100 (i.e. 1 mg of enzyme for 100 mg of substrate), it requires about 18 h for complete digestion of the IgM molecules at 37 °C [18]. However, in the absence of FBS, there are two substrates whose hydrolysis is catalysed by trypsin: the IgM antibody and the BSA present in the sample. The purified IgM used in these experiments has 1.0 g l^{-1} of IgM in 1.0% (by volume) BSA solution. Hence, in the reaction mixture for every milligram of IgM present there will be 10 mg of BSA present. The rate of reaction for the two substrates that are catalysed by the same enzyme is given by [19]

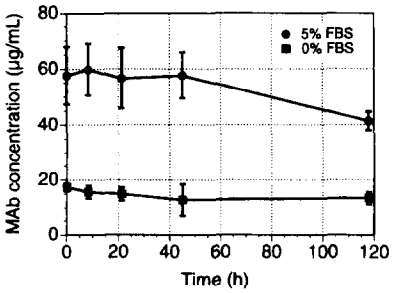

Fig. 1. Variation in ELISA titres with time for proteolysis experiments.

$$\frac{d[MAb]}{dt} = \frac{k_{MAb}e_0[MAb]/K_{MAb}}{1+[MAb]/K_{MAb}+[BSA]/K_{BSA}} \quad (7)$$

where e_0 is the enzyme concentration, k_{MAb} is the kinetic rate constant of the enzymatic reaction with the antibody and K_{MAb} and K_{BSA} are the Michaelis–Menten saturation constants for the two substrates, MAb and BSA respectively. The molar ratio of IgM to BSA in the reaction mixture is 1:128.6 (taking the molecular weights of IgM and BSA to be 900 kDa and 70 kDa respectively). Hence, from Eq. (7) it can be seen that initially the rate of proteolysis of IgM will be less than 1% of that in the absence of BSA as the typical values of K_{MAb} and K_{BSA} are in the range of 2–10 mmol l^{-1} [19]. By considering the first substrate to be BSA it can be readily seen that initially the rate of proteolysis of BSA will not be significantly affected by the presence of IgM. However, as the BSA is progressively depleted owing to proteolysis, the rate of proteolysis of IgM should increase at later times. This explains the observed trend for the proteolysis experiments in the absence of FBS. However, the changes in ELISA titres were rather insignificant for the reaction mixture containing 5% FBS. Hence, it can be inferred that FBS has some inhibitory effect on the proteolysis reaction. This may possibly be due to the presence of α_2-macroglobulin and α_1-antitrypsin protease inhibitors in FBS [3,20]. From these experimental results, the possibility of higher ELISA titres in the death phase of a batch culture being due to proteolysis in either the serum-free or the serum-supplemented can be ruled out.

6. Conclusions

The reactions in indirect ELISA assays have been analysed from a theoretical point of view. The speculation in the literature about the possibility of increase in ELISA titre due to proteolysis was examined. From the theoretical analysis it was concluded that for an antibody whose proteolytic fragments have association constants for the reaction with antigen and the reaction with enzyme-conjugated antibody nearly same as those of the intact antibody itself, the ELISA titre cannot increase as a result of proteolysis. The experimental results with IgM antibody led us to conclude that proteolysis cannot enhance the ELISA titre in a hybridoma cultures with serum-free medium as well as with medium containing FBS.

Appendix A: Nomenclature

A	total amount of antibody present in the well (mol)
A_i	total amount of antibody fragments with valence n_i in the well (mol)
C	total amount of enzyme-conjugated IgG present in the well (mol)
e_0	concentration of enzyme (mol l^{-1})

E amount of enzyme bound to the antigen–antibody complex in ELISA plate well (mol)

E_m amount of enzyme bound to the antigen–antibody complex in ELISA plate well for proteolysed sample (mol)

g number of antigenic determinant sites in heavy and light chains per "bound" antigen binding site

g_i number of antigenic determinant sites in heavy and light chains per "bound" antigen binding site in ith proteolytic fragment

I difference in absorbance between the standard sample well and the blank solution well (absorbance units)

k_m Michaelis–Menten reaction rate constant (l mol^{-1} s^{-1})

k_{MAb} kinetic rate constant in Eq. (7) (h^{-1})

K_{12} association constant for antigenic determinant site and antigen binding site reaction (l mol^{-1})

K_{ge} association constant for reaction between enzyme-conjugated IgG and the antigenic determinant site in heavy and light chains of the antibody (l mol^{-1})

K_m Michaelis–Menten constant (mol l^{-1})

K_{MAb} Michaelis–Menten constant for MAb (mol l^{-1})

K_{BSA} Michaelis–Menten for BSA (mol l^{-1})

n valence of the antibody

n_e valence of the enzyme-conjugated IgG molecule

n_i valence of the ith fragment of antibody

N number of antigen binding fragments produced by proteolysis including the intact antibody molecule

P total amount of antigen molecules present on the well surface (mol)

s valence of the antigen molecules

S concentration of the substrate (mol l^{-1})

t time (h)

V volume of the reaction mixture per well (l)

X amount of coloured protein produced in the well (mol)

Greek letters

γ amount of enzyme per "bound" antigen binding site of enzyme-conjugated IgG (mol)

ξ amount of antigenic determinant sites bound to the antigen binding sites in intact antibody (mol)

ξ_i amount of antigenic determinant sites bound to the antigen binding sites in the ith proteolytic fragment (mol)

ξ_m amount of antigenic determinant site bound to the antigen binding sites in a proteolysed sample (mol)

ϕ proportionality constant in Eq. (6) (absorbance unit mol^{-1})

References

[1] A.M. Campbell, *Monoclonal Antibody Technology*, Elsevier, Amsterdam, 1984.

[2] K. Sikora and H.M. Smedley, *Monoclonal Antibodies*, Blackwell, London, 1984.

[3] J.W. Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, London, 1986.

[4] E.D. Day, *Advanced Immunochemistry*, Williams and Wilkins, Baltimore, MD, 1972.

[5] R.S. Nezlin, *Structure and Biosynthesis of Antibodies*, Consultants Bureau, New York, 1977.

[6] W.J. Herbert, Passive haemmagglutination with special reference to the tanned cell technique, in D.M. Weir (ed.), *Handbook of Experimental Immunology*, Blackwell, London, 1978.

[7] R.B. Kratje, W. Lind and R. Wagner, *J. Biotechnol., 32* (19??) 107–125.

[8] E.J. Schlaeger, B. Eggimann and A. Gast, *Dev. Biol. Standard, 66* (1987) 403–408.

[9] M.C. Flickinger, C. Goebel, T. Bibila and S. Boyce-Jacino, *J. Biotechnol., 22* (1992) 201–226.

[10] R. van Erp, M. Adorf, A.P.G. van Sommeren, O.T. Schonherr and T.C.J. Gribnau, *J. Biotechnol., 20* (1991) 235–248.

[11] R. van Erp, M. Adorf, A.P.G. van Sommeren, O.T. Schonherr and T.C.J. Gribnau, *J. Biotechnol., 20* (1991) 249–261.

[12] J.-W. Bloemkolk, M.R. Gray, F. Merchant and T.R. Mosmann, *Biotechnol. Bioeng., 40* (1992) 427–431.

[13] D.E. Martens, C.D. de Gooijer, C.A.M. van der Velden-deGroot, E.C. Beauvery and J. Tramper, *Biotechnol. Bioeng., 41* (1993) 429–439.

[14] P.G. Sriram, Hybridoma cultures: the proteolytic degradation of immunoglobulin and dynamic modelling, *Ph.D. Dissertation*, University of Calgary, Calgary, 1995.

[15] R.F. Ashman and H. Metzger, *J. Biol. Chem., 244* (12) (1969) 3405–3414.

[16] M. Katz and A.M. Pappenheimer, Jr., *J. Immunol., 103* (1969) 491–495.

[17] H.A. Sober, *Handbook of Biochemistry: Selected Data for Molecular Biology*, Chemical Rubber Company, Cleveland, OH, 1968.

[18] D.R. Stanworth and M.W. Turner, Immunochemical analysis of immunoglobulin and their subunits, in D.M. Weir (ed.), *Handbook of Experimental Immunology*, Blackwell, London, 1978.

[19] J.E. Bailey and D.F. Ollis, *Biochemical Engineering Fundamentals*, McGraw-Hill, New York, 1986.

[20] D.L. Clemm, Scale-up of protein production in a stirred bioreactor, in D.R. O'Reilly, L.K. Miller and V.A. Luckow (eds.), *Baculovirus Expression Vectors, a Laboratory Manual*, Freeman, New York, 1992, pp. 241–256.