PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODY TO RECOMBINANT α-AMYLASE

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Four hybridoma cell lines that secreted monoclonal antibodies against thermostable α-amylase produced by a recombinant *Escherichia coli* were established. One of the clones, designated 16-3F, was quite stable with good growth in DF·ITES serum-free medium and high production of the antibody. Cell growth was independent of insulin, ethanolamine and selenite, but it strongly depended on transferrin. In the serum-free media the specific antibody production rate was almost constant irrespective of cell growth. Repeated batch culture in DF medium was found to be promising for antibody production after cell density was increased in DF·ITES medium. The affinity constant between the antibody and the antigen measured by a solid-phase ELISA was 1.3×10^8 dm^3/mol. The antibody did not bind four kinds of α-amylase derived from other organisms.

Introduction

Monoclonal antibodies established by hybridoma techniques have been extensively used in therapy, as diagnostics, and as a tool for purification and characterization of physiologically active substances. The advantages of the monoclonal antibody compared with its polyclonal counterpart reside in obtaining a large amount of homogeneous antibody and selecting an adequate monoclonal antibody for a particular use by selection of hybridoma cell lines. Thus, once a monoclonal antibody against physiologically active substance is selected, it provides tremendous advantages for purification of the substance.

In this paper, we report the establishment of hybridoma cell lines secreting a monoclonal antibody against thermostable α-amylase produced by a recombinant *Escherichia coli*. Production of the monoclonal antibody by 16-3F cell line, one of the hybridomas established in this study, under various medium conditions and the basic properties of the antibody are described.

1. Experimental

1.1 Animals and cell line

BALB/c mice were obtained from Shizuoka Animal Lab. BALB/c mouse myeloma cell line NS-1 (P3·NS-1/1·Ag4·1) was a kind gift of Dr. R. Ueda of Aichi Cancer Center.

1.2 Purification of α-amylase

E. coli HB101/pHI301, which harbored a plasmid[9] encoding *Bacillus stearothermophilus* α-amylase, was cultured in L-broth medium (10 kg/m^3 polypeptone, 5 kg/m^3 yeast extract, 5 kg/m^3 NaCl, 10 kg/m^3 glucose, and 0.1 kg/m^3 ampicillin, pH 7.0) by using a jar fermentor. The thermostable α-amylase was purified by the method of Tsukagoshi *et al.*[9] with a slight modification. The *E. coli* cells suspended in 0.05 mol/dm^3 Tris-HCl buffer pH 7.5 were disrupted with a sonicator (Ohtake Works Co.). After centrifugation, the supernatant was heated at 75°C in the presence of 0.01 mol/dm^3 $CaCl_2$ for 15 min. Denatured proteins were removed by centrifugation, and then proteins in the supernatant were precipitated in 55% saturated ammonium sulfate solution. After the precipitate was collected, proteins were dissolved in 0.05 mol/dm^3 Tris-HCl buffer pH 7.5 and the solution was dialyzed against two changes of the same buffer (1 dm^3). The solution was applied to a column of Sephadex G-75 (Pharmacia Fine Chemicals Co.) equilibrated with the same buffer. Eluted α-amylase fraction was purified to homogeneous state, which was checked by SDS-PAGE described below, and used as the antigen.

1.3 Immunization

Four 8-week-old female BALB/c mice were immunized by intraperitoneal injection of α-amylase. For this, 100 μg of purified α-amylase in 0.05 mol/dm^3 sodium-potassium phosphate buffer containing 0.14 mol/dm^3 NaCl pH 7.4 (PBS) was emulsified with the same volume of Freund's complete adjuvant in a total volume of 0.5 cm^3. Booster injection of 50–100 μg of the antigen was administered in the same manner at 2 and 4 weeks from the first injection. The antigen was emulsified with Freund's incomplete ad-

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juvant at the final booster. The sera from the immunized mice were tested for the formation of the antibody against the α-amylase by a solid-phase ELISA described below. Their spleen cells were isolated for cell fusion at three days after the final booster.

1.4 Cell fusion and hybridoma screening

Cell fusion and hybridoma screening were basically performed by the procedures described by Oi *et al.*[8] and Iwasaki *et al.*[4] Spleen cells were suspended in serum-free NS-1 medium (10.2 kg/m^3 RPMI1640 (Nissui Co.), 2.0 kg/m^3 $NaHCO_3$, 0.3 kg/m^3 L-glutamine, 50,000 U/dm^3 potassium penicillin G, and 50 g/m^3 streptomycin sulfate). In regular NS-1 medium, 10% (v/v) fetal calf serum (FCS; M.A. Bioproducts Co.) was supplemented. Then the spleen cells were fused with NS-1 mouse myeloma cells suspended in the same medium using polyethylene glycol 4000 (Merck AG). Spleen cells and myeloma cells was 5 to 1. The average number of spleen cells obtained from a mouse was about 1×10^8. The fusion mixture was replaced to selective HAT medium (NS-1 medium plus 100 mmol/m^3 hypoxanthine, 0.4 mmol/m^3 aminopterine, and 16 mmol/m^3 thymidine). Cells were seeded into 96-well microtiter culture plates. One well contained 0.2 cm^3 cell suspension (2×10^5 cells/well). BALB/c thymocytes or intraperitoneal cells were used as feeder cells. After 2–3 weeks of inoculation, supernatants of hybridoma growing in the wells were tested for the specific antibody production by the solid-phase ELISA. From antibody-positive wells, cells were transferred in 24-well tissue culture plates containing the feeder cells, and cultured with HT medium (NS-1 medium plus 100 mmol/m^3 hypoxanthine and 16 mmol/m^3 thymidine). At the same time, cloning by a limiting dilution method[4,8] and screening by the solid-phase ELISA were repeated to establish a hybridoma cell line producing the monoclonal antibody against α-amylase.

1.5 Cell culture for antibody production

Hybridoma cells were cultured in 100 mm petri dishes (Corning Glass Co.) containing 10 cm^3 medium. In all experiments, the dishes were incubated at 37°C in a humidified mixture of 5% CO_2 and 95% air. As serum-free medium, DF medium (5.24 kg/m^3 Dulbecco's MEM (Gibco Co.), 5.58 kg/m^3 Ham's F12 (Gibco Co.), 3.58 kg/m^3 Hepes buffer (Dojin Co.), 1.2 kg/m^3 $NaHCO_3$, 100,000 U/dm^3 potassium penicillin G, and 90 g/m^3 streptomycin sulfate) and DF·ITES medium[7] (DF medium plus 5 g/m^3 insulin, 35 g/m^3 transferrin, 1.2 cm^3/m^3 ethanolamine, and 4.3 mg/m^3 sodium selenite) were used. At the time of medium change, cells were centrifuged at 1,000 rpm and all cells were resuspended in the fresh medium.

1.6 Solid-phase ELISA

A solid-phase ELISA was used to detect antibody in the supernatants of hybridoma cell cultures or to determine antibody concentration in culture media. The method was basically the same as reported by Engvall *et al.*[2] Flexible microtiter plates (96 wells; Becton Dickinson Co.) were coated with 2 μg/well of purified α-amylase (10 g/m^3 in 0.05 mol/dm^3 carbonate buffer, pH 9.5) for 16 h at 4°C. After the plates were coated with 0.2% (w/v) bovine serum albumin in PBS, 0.12 cm^3 of test samples was added to each well and the plates were allowed to stand for 30 min. The antibody was detected by using goat anti-mouse IgG conjugated to horseradish peroxidase (Cooper Biomedical Co.) and *o*-phenylenediamine as the substrate. The antibody concentration was estimated by measuring absorbance at 492 nm. The antibody produced by 16-3F in DF medium was purified by a DEAE-TOYOPEARL (Tosoh Co.) column and used as the standard.

In an experiment to evaluate the antigenity of the antibody, four kinds of α-amylase preparations (from *Bacillus subtilis*, *Aspergillus oryzae*, barley and human saliva; all of them were purchased from Sigma Chem. Co.) were fixed to wells of a microtiter tray, and purified antibody solution (20 g/m^3) was added to each well. The antibody adsorbed to α-amylase was detected by peroxidase conjugated goat anti-mouse IgG.

1.7 Measurement of affinity constant

The antigen in PBS at various concentrations (4.6–41 nmol/dm^3) was mixed with a constant concentration (6.5 nmol/dm^3) of the antibody. The concentration of the antibody used was determined within such a range that the absorbance of ELISA samples was linear against the initial concentration of the antibody. The mixture was incubated at 4°C for 16 h, then at 20°C for another 2 h. The concentration of the free antibody in the mixture was determined by the solid-phase ELISA. The concentrations of the bound antibody and the free antigen were calculated from the concentration of the free antibody. The affinity constant was calculated by the Scatchard plot[3] based on the following equation, derived from equilibrium between the antigen and the antibody:

$$B/F = KA_0 - KB$$

where B is the bound antibody, F is the free antigen, A_0 is the total antibody concentration and K represents the affinity constant.

1.8 Other analytical methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10%gel) was performed to check the protein composition in culture media as described by Laemmli.[6] The proteins in sample solution were reduced by 2-mercaptoethanol before the electrophoresis. After the electrophoresis, gel was stained with a 0.1% (w/v) Coomassie brilliant blue R

solution and destained with a 7% (w/v) acetic acid solution.

The number of cells was determined using a hemacytometer with the trypan blue dye exclusion method. Activity of α-amylase was measured by the method reported by Tsukagoshi *et al.*[9]

2. Results and Discussion

2.1 Hybridoma producing monoclonal antibody against α-amylase

Spleen cells from the four mice immunized with thermostable α-amylase were combined and fused to NS-1 myeloma cells. Growth of hybridomas was observed in 823 wells out of 1356 wells. Supernatants of hybridoma growing in the wells were tested for specific antibody production by the solid-phase ELISA. Twenty-two cultures were found to be positive for the antibody production. The hybridomas of positive wells were transferred and cultured in 24-well tissue culture plates containing 1 cm^3-HT medium per well for freeze stocks. At the same time, the monoclonal antibody-producing cells were isolated by repeated cloning by a limiting dilution and screening by the solid-phase ELISA. After several cloning procedures, four cloned hybridomas (16-3F, 15-6D, 34-11F and 43-6C) were established as hybridoma cell lines producing monoclonal antibody against the α-amylase.

Table 1. Comparison of antibody production

Clone	Absorbance at 492 nm	Antibody concentration* [μg/cm^3]
15-6D	2.37	1.0
16-3F	3.42	102
34-11F	3.03	7.8
43-6C	2.47	1.5

* Antibody concentration was evaluated with purified 16-3F antibody as standard.

Table 1 shows a comparison of antibody concentrations when hybridomas were cultured in DF·ITES medium for 3 days with an initial concentration of 5×10^5 cells/cm^3. Antibody production of 16-3F was the highest among them, and 34-11F, 43-6C and 15-6D produced less in that order (production by 16-3F was about 100-fold that by 15-6D). Growth of 16-3F in the serum-free medium was the fastest and the other clones grew more slowly in the order 15-6D, 34-11F and 43-6C. From these results, we selected hybridoma 16-3F as a suitable cell line to prepare a large amount of monoclonal antibody.

2.2 Cultivation in various media of hybridoma 16-3F

To obtain monoclonal antibody, *in vivo* production by injecting hybridoma into mice has been widely used. However, a large amount of impurity is contained in the ascitic fluid of the injected mouse, and complete purification of antibody from the fluid is laborious and expensive.[1] The same difficulty of purification was observed when the hybridoma was cultured in a serum-containing medium. Therefore, hybridoma cell culture and antibody production in a serum-free medium are desirable to obtain a large amount of antibody with a simple purification procedure.

Figure 1 shows time-courses of viable cells, cell viability, antibody concentration and specific antibody production rate of 16-3F in three media for batch culture. In these cultures, the cells grown in HT medium were inoculated after washing the cells with each medium. Maximum viable cells obtained in HT medium containing 10% FCS in 2–3 days was 1.7×10^6 viable cells/cm^3 (3.5 times the initial cell density),

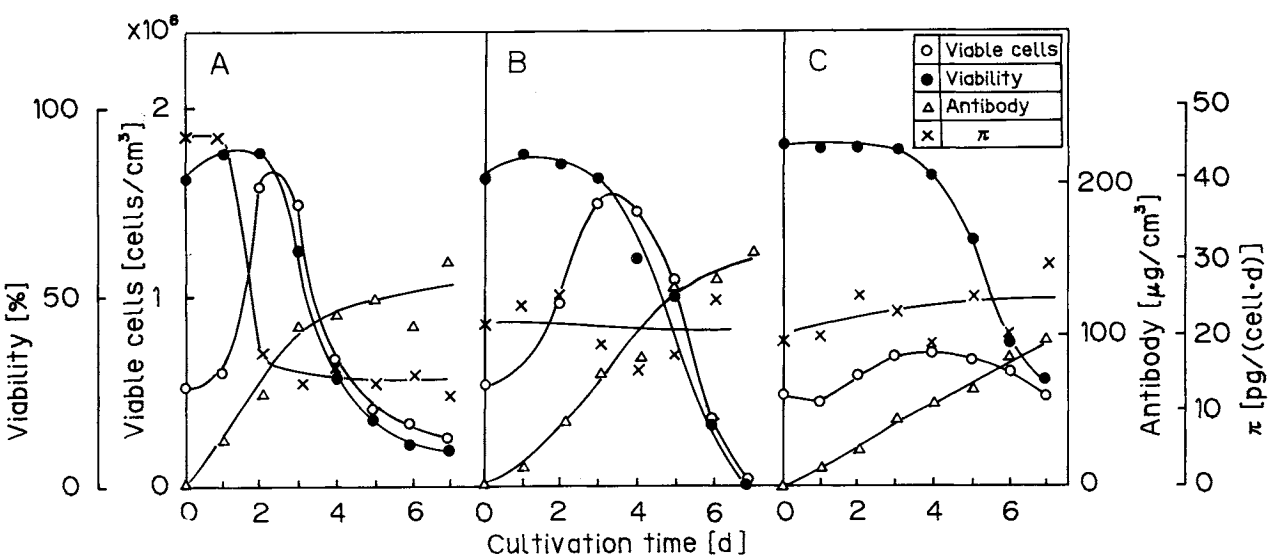

Fig. 1. Cell growth and antibody production of hybridoma 16-3F in HT (A), DF·ITES (B) and DF (C) media

then the figure dropped rapidly due to shortage of nutrients or inhibition by metabolites such as lactate or ammonium. In DF·ITES serum-free medium, maximum cell density (1.5×10^6 viable cells/cm^3) was obtained in 3–4 days, although cell growth was slightly slower. In DF medium, on the other hand, the cells grew very slowly for several days and thereafter the viable cell count decreased gradually. Specific growth rates in the logarithmic growth phase in HT, DF·ITES and DF media were 1.07, 0.55 and 0.16 d^{-1}, respectively.

In HT medium, the specific antibody production rate, π, was high in the early stage of culture and decreased to about 15 pg/(cell·d) as shown in Fig. 1A. On the other hand, it was almost constant in serum-free DF·ITES and DF media irrespective of cell growth, as shown in Fig. 1B and 1C. The values were almost the same.

As shown in Fig. 1B and 1C, insulin (I), transferrin (T), ethanolamine (E) and selenite (S) affected the cell growth. **Table 2** shows effects of these components on cell growth. Insulin, ethanolamine and selenite did not show profound effects on either specific growth rate or maximum cell concentration. However, the cell growth depended strongly on transferrin. To obtain maximum cell concentration, 35 g/m^3 of transferrin was needed.

Table 2. Effect of transferrin on the growth of hybridoma 16-3F

Medium	Transferrin concentration [g/m^3]	Maximum cell concentration [cells/cm^3]	Specific growth rate [1/d]
DF	0	7.8×10^5	0.13
DF·IES*	0	9.5×10^5	0.17
DF·ITES*	8	1.1×10^6	0.41
	17	1.2×10^6	0.43
	35	1.4×10^6	0.50
	70	1.5×10^6	0.55
DF·T	35	1.4×10^6	0.48

Initial cell concentration was 5×10^5 cells/cm^3.
* Concentrations of I, E and S were 5 g/m^3, 1.2 cm^3/m^3 and 4.3 mg/m^3, respectively.

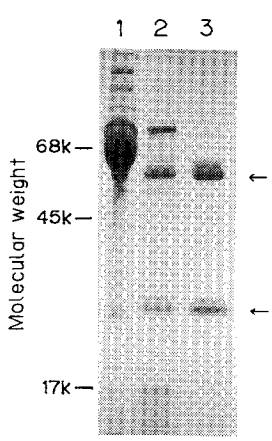

Fig. 2. SDS-polyacrylamide gel electrophoresis patterns of anti-α-amylase monoclonal antibody produced in three kinds of media after 7 days
1, HT medium; 2, DF·ITES medium; 3, DF medium. Figures on the left side show the position of standard proteins: bovine serum albumin (M.W. 68,000); ovalbumin (M.W. 45,000); β-lactogloblin (M.W. 17,000). Arrow-heads on the right side show the bands of the H- and L-chain of the antibody

Figure 2 shows SDS-PAGE patterns of supernatants obtained from 16-3F cultures in three kind of media after 7 days. Bands of H-chain (M.W. 55,000) and L-chain (M.W. 25,000) derived from the antibody were almost the same density for all media. Many bands from serum proteins were observed in HT medium sample. In the cases of samples from DF·ITES and DF media, the antibody protein showed major bands, but the band of transferrin (M.W. 74,000) was also observed in DF·ITES sample. The antibody from the serum-free media could be purified to homogeneous state by an ion-exchange chromatograph (DEAE-TOYOPEARL) with 0–0.5 mol/dm^3 NaCl linear gradient (data not shown). However, the chromatographic operation was easier in DF sample because contaminating proteins were fewer than those in DF·ITES sample. The antibody from HT medium was not purified to homogeneous state by ion-exchange chromatography (data not shown).

2.3 Production of antibody by hybridoma 16-3F

Based on the commercial prices of the medium components, the relative costs of HT and DF·ITES media to that of DF medium were evaluated as 5.9- and 4.2-fold respectively. Thus, production of the antibody in HT medium is not promising because the cost of the medium is the highest and the purified antibody could not be obtained by single-step ion-exchange chromatography. However, production of the antibody in DF·ITES medium is not promising either, because the cost of the medium is also high and

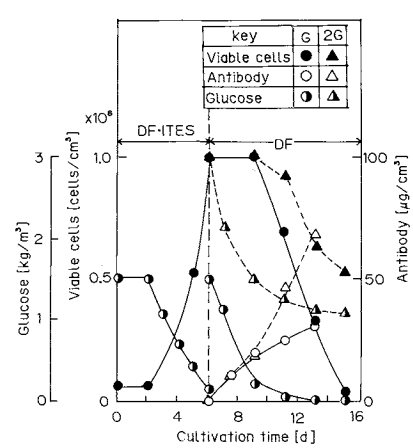

Fig. 3. Antibody production by the two-step culture at different glucose concentrations
Initial glucose concentrations were 1.5 kg/m^3 for G and 3.0 kg/m^3 for 2G

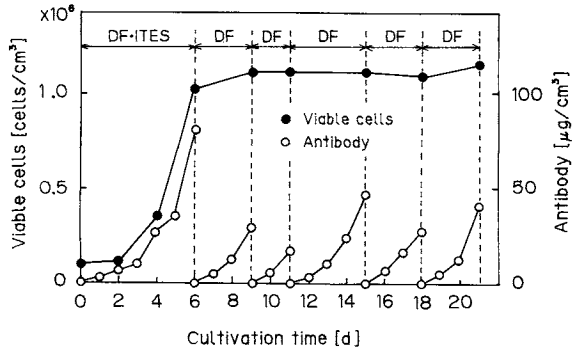

Fig. 4. Antibody production by the two-step repeated culture with DF medium

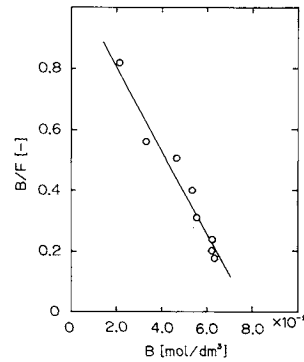

Fig. 5. Scatchard plot for binding of thermostable α-amylase to 16-3F antibody

the specific antibody production rate was almost the same as that in DF medium. The feasibility of a two-step culture, i.e. cell growth in a rich but expensive medium and subsequent antibody production in a cheap one, was examined.

As shown in Fig. 1C, the specific antibody production rate of the hybridoma in DF medium was comparable to that in DF·ITES medium although cell growth was very slow. Therefore, cells grew to a certain density in DF·ITES medium and then the medium was changed to DF medium to produce the antibody. At the medium change, all cells were transferred to fresh medium after centrifugation. **Figure 3** shows the result. The medium was changed when viable cells reached 1×10^6 cells/cm^3 in DF·ITES medium. After glucose was consumed in DF medium, viable cells and the antibody production rate decreased gradually. Decrease of viable cells was also observed after 3–4 days culture when the initial glucose concentration was doubled. Thus, DF medium should be exchanged after 3–4 days culture. When the medium was changed after viable cells reached 1.5×10^6 cells/cm^3 in DF·ITES medium, the cell density in DF medium decreased just after the medium change (data not shown). Therefore, after cell density reached 1×10^6 cells/cm^3 in DF·ITES medium, the medium was switched to DF medium and repeated batch culture with DF medium was begun.

Time-courses of viable cell density and antibody concentration are shown in **Fig. 4**. It was possible to maintain cell density at the initial value (1.1×10^6 cells/cm^3) by changing the medium every 2–4 days. During the culture, DF medium was exchanged so that glucose in the medium was not depleted. Average specific antibody production rate at the medium exchange time was 17 pg/(cell·d), which was 75% of that in batch culture (Fig. 1C). This two-step culture was found to be promising for antibody production over a long period.

2.4 Characteristics of antibody produced by hybridoma 16-3F

The purified antibody was used to characterize its properties. **Figure 5** shows the Scatchard plot of the binding of α-amylase to 16-3F antibody measured by the solid-phase ELISA. The affinity constant calculated from the slope was 1.3×10^8 dm^3/mol. The 16-3F antibody showed high affinity with the thermostable α-amylase. This value showed good agreement with the value[5] (1.1×10^8 dm^3/mol) obtained from the equilibrium between the thermostable α-amylase and immuno-adsorbent (the 16-3F antibody was immobilized to Sepharose 4B).

Table 3. Antigenity of α-amylase preparations against 16-3F antibody

Origin	Absorbance at 492 nm	Antibody concentration* [μg/cm^3]
E. coli HB101/pHI301	2.42	92.8
B. subtilis	0.06	0.05
A. oryzae	0.02	0.03
Barley	0.07	0.07
Human saliva	0.12	0.10

* Antibody concentration represents the concentration of 16-3F antibody equivalent to the absorbance value.

The solid-phase ELISA was used to investigate whether the antibody recognized α-amylase from other species. **Table 3** shows absorbance at 492 nm of oxidized substrate of peroxidase in the well. α-Amylases other than that from *E. coli* HB101/pHI301 employed as antigen did not bind the antibody produced by 16-3F at all. Not were α-amylases from organisms other than *E. coli* HB101/pHI301 adsorbed to the column of Sepharose 4B to which the antibody was immobilized (data not shown). These results showed again the higher specificity of the monoclonal antibody.

E. coli has been widely used as host strain in gene engineering, and foreign gene products are usually accumulated in the cells. This makes it difficult to purify products such as hormones or growth factors to the degree necessary for drug use. Immuno-affinity

chromatography using monoclonal antibody allows the purification step to be simplified, because of its high specificity and strong affinity. In our previous paper[5] we showed the effectiveness of immunoaffinity chromatography in the purification of protein produced by gene engineering.

Conclusions

Four hybridoma cell lines that secrete monoclonal antibodies against thermostable α-amylase produced by *E. coli* HB101/pHI301 were established. The 16-3F cell line showed the best cell growth and antibody production among the four clones. In serum-free media, the specific antibody production rate was almost constant and was independent of cell growth. Cell growth depended on transferrin. Two-step culture, i.e. cell growth in a rich but expensive medium and the subsequent antibody production in a cheap one, was found to be feasible for antibody production. The affinity constant between the 16-3F antibody and the antigen was 1.3×10^8 dm^3/mol. The antibody did not bind to four kinds of α-amylase other than the antigen.

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Nomenclature

A_0	=	total antibody concentration	[mol/dm^3]
B	=	bound antibody concentration	[mol/dm^3]
F	=	free antigen concentration	[mol/dm^3]
K	=	affinity constant	[dm^3/mol]
P	=	antibody concentration	[μg/cm^3]
t	=	culture time	[d]
X	=	viable cell concentration	[cell/cm^3]
π	=	$(1/X)\cdot(dP/dX)$ = specific antibody production rate	[pg/(cell·d)]

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