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ABSTRACT

A collection of monoclonal antibodies was raised against bovine plasminogen by immunizing mice with purified plasminogen. More than 300 positive clones were obtained in 1 fusion experiment (1300 hybrids tested). Four types of antibodies were characterized through their relative binding to plasmin and plasminogen in ELISA. About one-third was strictly specific to plasminogen; the selectivity of this group was confirmed by immunoblots and BIA-core analysis. Cross-reactivity against horse, human, pig, sheep, rabbit, and bovine plasminogens was tested; 41% were strictly bovine specific.

INTRODUCTION

PLASMINOGEN is a component of the fibrinolytic system and is the precursor of a serine proteinase, plasmin, which displays an activity similar to that of trypsin.[1] Plasmin-plasminogen activation systems are involved in a number of physiological and pathological processes. In particular, they take part in involution of mammary gland at the end of lactation.[2] Activation of plasminogen (M_r ~90K) to plasmin by urokinase involves two proteolytic steps:[3] first a cleavage (Lys-77–Arg-78) causes the release of a 7-kDa peptide at the N-terminal end of the molecule, referred to as the preactivation peptide. Providing that the preactivation peptide has already been released, a second cleavage at Arg-557–Ile-558 results in the formation of plasmin. Plasmin is, thus, a two-chain molecule with a heavy chain (A; M_r 60K) and a light one (B; M_r 20K) connected by a disulfide bond.[3] Important conformation changes are induced during activation of plasminogen to plasmin.[4] Primary structures of plasmin and plasminogen have been established in human and bovine species[5]; the overall identity between plasminogens of these two species is 78%.

The plasminogen–plasmin system is exported through the mammary gland to the milk and therefore may affect the properties of milk and milk products during storage.[6] This enzyme is thus partially responsible for proteolysis of dairy products. In bovine milk this protein degrades parts of β-casein,[7] causing the appearance of γ-caseins, and it has been demonstrated that it is also responsible for the αS2-casein hydrolysis.[8] These modifications involve an increase in soluble nitrogen, resulting in a decrease in cheese yield.

During ultra high temperature (UHT) treatment of milk and Swiss-type cheese production, most of the enzymes are partially or totally inactivated as a result of heating. Plasmin, on the contrary, holds out against this thermic treatment and its activity even seems to be increased.[9] Determination of potential plasmin activity by differential titration of plasminogen and plasmin is thus important in dairy technology.

Plasmin activity can be determined by different enzymatic methods,[10,11] but the results found in the literature vary greatly owing to the fixation of this enzyme to the caseins.

The total amount of plasminogen and plasmin can be titrated using cross-reacting polyclonal antibodies in an enzyme-linked immunosorbent assay (ELISA).[12]

To make a differential assay available, we present, in this work, production and characterization of monoclonal antibodies strictly specific to bovine plasminogen.

MATERIALS AND METHODS

Production of hybridomas

BALB/c mice were injected intraperitoneally with 100 μg of plasminogen purified as previously described[13,14] and emulsi-

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fied in Freund's complete adjuvant. After a rest period of 1 month and 3 days before fusion, the mice were further immunized by an intraperitoneal injection of 100 μg of the antigen without any adjuvant.

Fusion of splenocytes with myeloma cells sp2/0-Ag14[15] was carried out with 1 ml of 45% polyethylene glycol 1000 (E. Merck AG, Darmstadt, Germany), following the procedure described by Köhler and Milstein.[16]

Supernatants of hybrid clones were assayed by direct ELISAs (see below). Secreting hybridomas were subcloned in thymocyte-conditioned medium by limiting dilution. Their monoclonal nature was checked at a confidence level of 95% according to Poisson's distribution.[17]

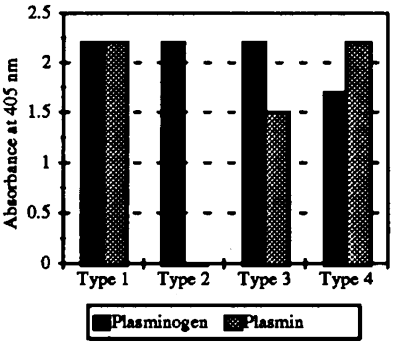

FIG. 1. The four different types of positive clones obtained.

Hybridoma screening and reactivity characterization

An ELISA for the detection of antibodies directed against plasmin and plasminogen in the culture supernatants was performed according to Scherrer and Bernard[18]; microtiter plates (Falcon Probind 3915; Becton Dickinson Labware, Oxnard, CA) were coated with 100 μl of plasmin (0.5 μg/ml) purified as previously described[14] or with 100 μl of plasminogen (0.5 μg/ml) in 0.1 M bicarbonate buffer, pH 9.6. Blocking of the remaining binding sites was performed with 1% gelatin (Sigma, St. Louis, MO). These plates were then filled with 100 μl per well of hybridoma supernatants diluted 1:2 in 0.1% Tween 20 in PBS (phosphate-buffered saline) for the selection and cloning procedure. Bound antibodies were quantified with goat anti-mouse IgG–alkaline phosphatase conjugate (Biosys, Compiègne, France) and *P*-nitrophenyl phosphate (PNPP) as color reagent. The color development was read at 405 nm in a Titertek Multiskan Autoreader (Flow Laboratories, Herts, England).

To characterize the antibodies against plasminogen of different species, the same test as described above was repeated on plates coated with plasminogen from six different sources: horse, rabbit, pig, sheep, human, and bovine (Sigma).

Reactivity of monoclonal antibodies toward preactivation peptide was checked by ELISA on Microtest flexible assay plates (Falcon 3912), following the same procedure as described above.

Isotyping of monoclonal antibodies

Monoclonal isotypes were determined using the ISO-STAT test (Sang Stat Medical, Menlo Park, CA).

Electrophoretic blotting procedure

Equimolar mixing of plasminogen and plasmin was electrophorsed[19] and transferred[20] to nitrocellulose with a Transblot apparatus (Hoefer Scientific Instruments, San Francisco, CA) for 1 hr and 30 sec at 50-V (10°C). The nitrocellulose sheet (Bio-Rad, Richmond, CA) was incubated in the following way:

1. At 37°C for 1 hr in 2% gelatin in PBS to block nonspecific binding of antibodies
2. At room temperature overnight with ascitic fluids at a dilution of 1:1000
3. With an anti-mouse IgG–alkaline phosphatase conjugate at a dilution of 1:2000 (Biosys), then with the enzyme substrate (equal volumes of 0.04% [w/v] Fast-Red TR salt in water and 0.6% [w/v] naphthol AS-MX phosphate disodium salt in 0.2 M Tris-HCl [pH 8] [Sigma].

BIA-core analysis

Real-time biospecific interaction analysis (BIA) based on surface plasmon resonance (SPR); Pharmacia, Piscataway, NJ) was used to characterize monoclonal antibody capacities toward plasmin and plasminogen.[21] Experimental conditions are described in Fig. 3.

RESULTS AND DISCUSSION

Production and classification of hybridomas

One fusion experiment yielded 309 positive clones from 1279 clones analyzed. From differential ELISA reactivities on plasmin and plasminogen, they could be classified into four types (Fig. 1):

Type 1: 63 clones share an identical absorbance with plasmin and plasminogen (absorbance plasminogen = absorbance ± 10%).
Type 2: 105 clones recognize only plasminogen in ELISA (absorbance plasmin < absorbance negative control).
Type 3:135 clones have a plasminogen absorbance higher than that of plasmin in ELISA (absorbance plasminogen > absorbance plasmin + 20%).
Type 4:6 clones have a plasmin absorbance higher than that of plasminogen as seen in ELISA (absorbance plasminogen + 20% < absorbance plasmin).

Characterization of plasminogen-specific antibodies

Type 2 antibodies were further studied to confirm their selectivity toward plasminogen. Monoclonal antibody 41(317) was chosen as a representative of this group.

Immunoblot analysis is presented in Fig. 2. Monoclonal antibody 41(37) gives only one band at ~88 kDa, corresponding

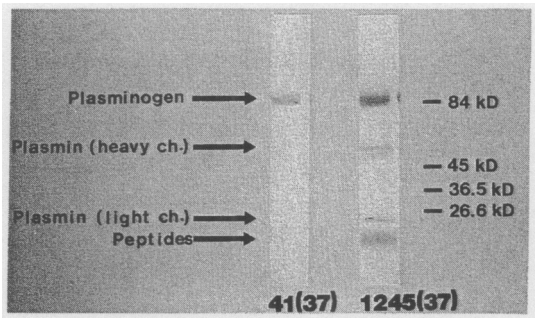

FIG. 2. Immunoblot analysis with antibodies 41(37) and 1245(37).

to plasminogen; in contrast, monoclonal antibody 1245(37) (type 1) recognizes four bands corresponding to plasminogen, light and heavy chains of plasmin, and autodegradation peptides.

BIA-core analysis (Fig. 3) demonstrated 41(37) to be a good capture antibody for plasminogen and confirmed that there was no recognition of plasmin in solution.

Reactivity for preactivation peptide (plasminogen N-terminal peptide) was tested by ELISA with three type 2 monoclonal antibodies [41(37), 553(51), and 1255(68)]. Only MAb 553(51) reacted with this peptide preparation when coated onto plastic.

Interpretation of monoclonal antibody specificity toward plasmin and plasminogen

Disappearance or appearance of some epitopes can signify modification of the three-dimensional structure.[22] A monoclonal antibody directed against an epitope that is present in both plasminogen and plasmin molecules can be fixed differently on these two antigens, if this epitope is not exposed in the same way.

We must determine whether these different types of monoclonal antibodies we have obtained against bovine plasminogen really correspond to the tool we are looking for. Type 1 seems to correspond to antibodies directed against an epitope that is present in both molecules and probably exposed in the same way on plasmin and plasminogen, or at least they seem similar when fixed and partially deformed on plastic-bottomed ELISA plates. About 90% of the type 1 monoclonal antibodies tested by immunoblotting seemed to recognize the heavy and light chains of plasmin. We can suppose that these monoclonal antibodies are directed against a conformational epitope comprising surface residues of both chains that come into close proximity by virtue of the folding of the polypeptide chain, but that are not necessarily directly linked by peptide bonds.

Type 2 can be explained in various ways. It is possible that these antibodies are directed against the N-terminal part of the plasminogen molecule, part of which is eliminated during the activation (release of the 7-kDa peptide). It is also possible that this epitope is present, but completely buried in the plasmin molecule and out of reach. This type seems to be the most interesting because it could possibly quantify, specifically, the plasminogen concentration in a dairy product sample, which would be impossible with a polyclonal antibody.

The difference observed between the plasmin and plasminogen absorbances by type 3 antibodies can be explained by the appearance or disappearance of some epitopes by modification during plasminogen activation. Some epitopes may not be reached as easily on the plasmin molecule by the antibody as on the plasminogen molecule. This difference can also be due to a difference in antibody affinity toward those two molecules.

Type 4 disappeared after subcloning and was not further studied.

Characterization of the antibodies toward bovine, human, pig, horse, rabbit, and sheep plasminogen

ELISA allowed characterization of 210 antibodies in accordance with their capacity to recognize the plasminogen of one to six different species (bovine, human, pig, horse, rabbit, and sheep).

Among the 210 anti-bovine plasminogen antibodies tested, 87 are strictly bovine specific whereas 90 cross-react with sheep, 40 with pig, 36 with human, 24 with horse, and only 7 with rabbit plasminogen (an antibody is, of course, able to cross-react with plasminogens of more than one species).

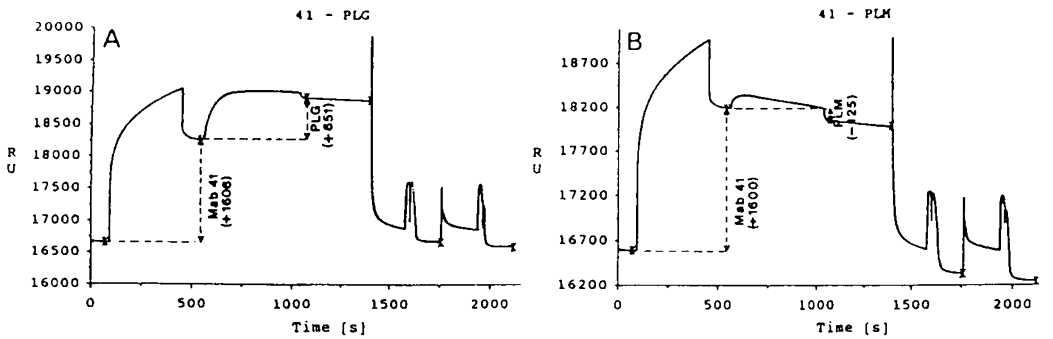

FIG. 3. BIA-core analysis of 41(37) interaction toward plasminogen (A) and plasmin (B). After immobilization of rabbit anti-mouse immunoglobulin Fc (RAM) on the dextran, the following sequential injections were performed (flow, 5 µl/min): 30 µl of monoclonal antibody 41(37) supernatant, 40 µl of 50 µg/ml purified plasminogen (A) or plasmin (B). The RAM biocaptor was then regenerated with two pulses of 1 M formic acid.

TABLE 1. SUMMARY OF CHARACTERISTICS OF THREE ANTI-PLASMINOGEN-SPECIFIC MONOCLONAL ANTIBODIES

Clone	*Isotype*	*Specificity toward plasminogen demonstrated by BIA-core analysis*	*Recognition of the preactivation peptide*	*Plasminogen(s) from other species recognized*
41 (37)	G_1	+	−	Bovine
553 (51)	G_1	ND	+	Bovine, pig, horse
1255 (68)	G_{2a}	+	−	Bovine, pig

Abbreviation: ND, not determined.

Two antibodies recognize the plasminogen of all six species, proving that there is at least one epitope common to the six molecules.

Only 36 antibodies of 210 recognize human plasminogen, although the overall identity between the sequences of these 2 molecules is 78%.[5]

Bovine and ovine plasminogens exhibit a strong identity, tending to demonstrate that when two species are closely related, the epitopic constitution of their plasminogens is similar.

CONCLUSION

According to their structural homology, we have observed a strong cross-reactivity between plasminogen and plasmin. Nevertheless, it was demonstrated that a monospecific monoclonal antibody can recognize two antigens that lack any structural identity. Cross-reactivity can be a function of the affinity between an antigen and an antibody, as demonstrated in the model of Campbell.[20]

The results on the supernatants, using ELISA and immunoblotting, show that we succeeded in producing at least three different monoclonal antibodies that recognize only plasminogen (type 2) (see Table 1). These monoclonal antibodies should allow the determination of the amount of plasminogen that is available for transformation into active plasmin in a milk sample.

We have also obtained a monoclonal antibody collection, which should give an idea of the total quantity of plasmin and plasminogen in a milk sample (type 1).

With this large family of monoclonal antibodies, it should be possible to observe the change in bovine plasminogen levels in milk as a function of time, to understand better the proteolysis in dairy products.

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