Monoclonal Antibodies as Probes for the Changes in Antigenicity of Bovine and Porcine Aspartyl Proteases with pH

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Two monoclonal antibodies, 918(4) and 139(7), directed against either bovine or porcine pepsin, respectively, were retained among 365 positive hybridoma clones. These monoclonal antibodies were characterized by using both indirect and sandwich ELISA. Characterization of these monoclonal antibodies was further performed by the biospecific interaction analysis (BIA-core analysis). Then, they were used as antigenic probes to study the changes in antigenicity of both bovine and porcine pepsins induced by pH. The results demonstrated the importance of the conformational change in both catalytic activities and antigenic determinant accessibility of bovine and porcine pepsins. Furthermore, our results suggest that changes in the conformation due to pH can be detected by specific monoclonal antibodies.

Keywords: *Monoclonal antibodies; bovine pepsin; porcine pepsin; antigenicity; pH*

INTRODUCTION

Aspartyl proteases (EC 3.4.23) are a group of proteolytic enzymes found in a wide range of species, from vertebrates to lower eukaryotes and retroviruses (Foltmann, 1981; Hsu et al., 1977; Katoh et al., 1987). They are all characterized by the presence of two aspartic acid residues at the active site and are inhibited by pepstatin, a pentapeptide naturally synthesized by strains of streptomyces (Umezawa et al., 1970). The best known source of aspartyl proteases is the stomach, which produces chymosin, gastricin, and pepsin. The availability of high-resolution crystal structures and the kinetic and specificity data of pepsin (EC 3.4.23.1) has led to its use as a model for studies of structure and function relationships of the aspartyl protease family (Abad-Zapatero et al., 1990; Sielecki et al., 1990). Animal proteases have been widely used in cheese manufacture. The shortage of calf stomachs in the world market has prompted a search for new sources of milk coagulants. Mixed proteases have been studied (Phelan et al., 1973), and a mixture of bovine and porcine pepsins often used as the so-called "fifty-fifty" coagulant has been used extensively in the cheese-making process (Nelson, 1975; Guinee and Wilkinson, 1992). Bovine and porcine pepsins consist of a 326 amino acid residue chain with a molecular mass of 36 kDa, and the overall homology between them is more than 80% (Tang et al., 1973; Foltmann and Pederson, 1977). Nevertheless, they differ in their optimal pH, catalytic and proteolytic activities, and some substrate specificities (Fox, 1969; Fruton, 1976; Andren et al., 1983). The role of hydrogen bonds in aspartyl proteases is to provide conformational stability in the catalytic apparatus. Their alteration at pH values higher than 6 causes irreversible decreases in catalytic activity and changes in conformation (Lin et al., 1992). Nevertheless, limited information is available concerning the pH dependence of the conformational changes of pepsin (Smith and Yada, 1991). No information is available concerning the use of monoclonal probes to study the pH dependence of the antigenicity (the capacity of antigens to react with their antibodies) of pepsin and its catalytic activity.

Monoclonal antibodies have been demonstrated to be powerful tools for studying biological molecular structure and function (Van Regenmortel, 1989). Because of their specificity and ease of use as compared to other methods, monoclonal antibodies have been increasingly used as immunologic probes for proteic conformational studies (Godinot et al., 1986; Collawn et al., 1988; Goldberg, 1991). In previous work (Boudjellab et al., 1998), we have demonstrated the difficulty of obtaining specific antisera to either bovine or porcine pepsin. The main objective of this study was to produce and select monoclonal antibodies that can specifically recognize porcine pepsin or bovine pepsin. The study reported here was also designed to demonstrate the usefulness of specific monoclonal antibodies as immunologic probes to further our understanding on how the pH affects the antigenicity of both porcine and bovine pepsins in relation to their conformational changes. The latter consequently led to the change in clotting activities (Lin et al., 1992; Smith and Yada, 1991). Thus, clotting activities were measured to estimate the change of conformation. This is apparently the first paper to elucidate the effect of pH on the antigenicity of both bovine and porcine pepsins by using monoclonal antibodies as antigenic probes.

MATERIALS AND METHODS

Materials. Crystalline porcine pepsin and poly(ethylene glycol) 1000 were purchased from Merck (AG, Darmstadt,

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Germany). Porcine pepsin was the highest grade commercially available and was used without further purification. Bovine pepsin was obtained from Granday (Beaune, France) and purified as indicated below. Complete and incomplete Freund's adjuvants were from Difco (Detroit, MI), RPMI 1640 and fetal calf serum (FCS) were from Gibco BRL (Grand Island, NY). Tween 20, *p*-nitrophenyl phosphate, diethanolamine, sulfate ammonium, pristane, hypoxanthine, and the goat Ig anti-mouse Ig (Fc) were purchased from Sigma Chemical Co. (St. Louis, MO). The isotype Ab STAT kit was obtained from Sang Stat Medical (Menlo Park, CA). DEAE Trisacryl LKB was purchased from Sepracor (Villeneuve-la-Garenne, France). Both the donkey IgG anti-mouse Ig and the goat IgG anti-rabbit Ig alkaline phosphatase conjugates were obtained from Biosys (Compiègne, France). Microtiter plates were from Nunc (Roskilde, Denmark). The microtiter ELISA autoreader EL 310 was from Bio-Tek Instruments (Winnoski, VT). The BIA-core instrument was purchased from Pharmacia Biosensor (Uppsala, Sweden). All other solvents and reagents were of analytical quality.

Rabbit antiserum directed against both porcine and bovine pepsins (Pp44) was produced previously (Boudjellab et al., 1998). In the present study, the crude antiserum Pp44 was used without any adsorption.

Methods. *Bovine Pepsin Purification.* Purified fractions of bovine pepsin were obtained by ion exchange chromatography on DEAE Trisacryl according to the method described by Collin et al. (1981). Briefly, a DEAE Trisacryl column (10 mm × 10 cm) was equilibrated with 0.025 M piperazine buffer, pH 5.3, at a flow rate of 1.3 mL/min. Commercial solution of bovine pepsin (1170 mg of active bovine pepsin/mL) diluted at 1/100 in the same buffer was used to load the column. Samples were eluted by a linear gradient of NaCl from 0 to 0.8 M. Bovine pepsin fractions were eluted at 0.45 M NaCl, and their purity was verified by electrophoresis on polyacrylamide gel−SDS (PAGE−SDS). Active bovine fractions were assessed by standard milk clotting activity at pH 5.3, and the final pure protease was dialyzed several times against 0.025 M piperazine buffer, pH 5.3, before freezing until use.

Determination of Enzymatic Activity. Both bovine and porcine pepsin solutions at different pH values were prepared in either 0.1 M citrate buffer for pH ≤ 6 or 0.5 M phosphate buffer for pH ≥ 6.5 (see Results and Discussion for more details). The enzyme solutions were then incubated for 30 min in a water bath at 30 ± 0.05 °C before use. Clotting time was determined according to the method described by Berridge (1952) and modified by Collin et al. (1977).

Production of Monoclonal Antibodies. Four female Balb/c mice (two for each antigen) were injected intraperitoneally with 100 μg of either bovine pepsin (4 times) or porcine pepsin (3 times) during a period of 12 weeks. The first injection was given with an equal volume of complete Freund's adjuvant and boosters with an equal volume of incomplete Freund's adjuvant. The last injection was given with an equal volume of saline. Three days after the final injection, spleen cells were removed and fused with myeloma cells (SP2/O-AG 14) (Shulman et al., 1978) at a ratio of 5:1 (spleen cells:myeloma) using poly(ethylene glycol) 1000 according to the procedure described by Nowinsky et al. (1979). Cell suspensions were then diluted with RPMI 1640 medium containing 10% FCS and 0.1 mM hypoxanthine to select hybridoma cells, which were divided in a 48-well plate for incubation at 37 °C with 5% CO_2.

Screening and Cloning of Hybridoma. Supernatants of hybrid clones were assayed for anti-bovine or porcine pepsins using both an indirect and a double antibody sandwich ELISA. Positive hybridoma were subcloned in macrophage (P388D1)-conditioned medium (RPMI 1640 containing 10% FCS) by limiting dilution as described by Nowinsky et al. (1979), and their monoclonal nature was checked at a confidence level of 95% according to Poisson's distribution (De Blas et al., 1981). The class and subclass of selected monoclonal antibodies were determined using the isotype Ab STAT test. The cloned hybridoma were grown in medium and injected into Balb/c mice primed with pristane, and antibodies were harvested as ascitic fluid. Ascitic fluids were then fractionnated by 40% saturation of ammonium sulfate precipitation and dialyzed.

ELISA Protocols. To prevent the selection of Mabs directed against only accessible epitopes when the antigens were adsorbed onto the solid phase, Schwab and Bosshard (1992) recommended complementing the indirect ELISA with another ELISA in solution. Thus, two ELISA methods were performed in order to detect specific monoclonal antibodies against bovine or porcine pepsins. For the screening and the cross-reaction tests, antigens were diluted in 0.05 M phosphate/0.15 M NaCl buffer, pH 7.4 (PBS).

(a) Indirect ELISA. Microplates were coated overnight at 4 °C with 100 μL of antigen (either bovine or porcine pepsin at 5 μg/mL) in 0.1 M sodium carbonate buffer, pH 9.6. For tests according to pH, microplates were coated with antigen solutions in citrate buffer for pH values ≤6 and in phosphate buffer for pH values ≥6.5. Plates were blocked with 0.5% gelatin in PBS containing 0.55 g of Tween 20/L (PBS-T) (w:v) and then 100 μL of hybridoma supernatants per well was added. Bound antibodies with bovine and/or porcine pepsin were detected with the donkey anti-mouse Ig alkaline phosphatase conjugate diluted in PBS (1:7000) and *p*-nitrophenyl phosphate disodium salt at 1 mg/mL in 0.1 M diethanolamine buffer, pH 9.8, as the color reagent. Plates were allowed to develop for 60 min at 37 °C. Absorbances were determined on an ELISA plate reader at 405 nm. Plates were washed four times with PBS-T after each step.

(b) Double Antibody Sandwich. This ELISA method was based on the reactivity of the monoclonal antibodies with antigens in solution. To avoid catalytic digestion of immunoglobulins, the catalytic site of pepsin was occupied with pepstatin. The clotting activity of porcine pepsin at the concentration of 405 μg/mL in the citrate buffer, pH 5.3, was inhibited by 92% after incubation in the presence of 1 μg/mL of pepstatin at 37 °C for 30 min.

Supernatants (100 μL) were coated onto the solid phase via a goat anti-mouse Ig (Fc). Plates were then blocked with PBS-T containing 2% gelatin. After a 4-cycle washing, plates were added with either bovine or porcine pepsin at 100 ng/mL diluted in either citrate buffer (pH ≤ 6.0) or phosphate buffer (pH ≥ 6.5) and incubated at 37 °C for 90 min. After another 4-cycle washing, each well had 100 μL of rabbit anti-porcine and anti-bovine pepsins (Pp44) added, which was diluted at 1/2000 in PBS. Bound rabbit antibodies with bovine and/or porcine pepsins were detected with a goat anti-Ig rabbit alkaline phosphatase conjugate, diluted at 1:7500 in PBS. Subsequent steps of this ELISA were similar to those for the indirect ELISA.

Biospecific Interaction Analysis (BIA). The BIA-core instrument was used to characterize Mabs against bovine or porcine pepsins at different pH values. Antibody−antigen interactions were monitored by surface plasmon resonance (Karlsson et al., 1991).

Rabbit anti-mouse Ig purified immunoglobulins (RAM) were covalently linked to the matrix gel via amino group coupling as recommended by Pharmacia Biosensor. The ability of antigens (both porcine and bovine pepsins inhibited by pepstatin) at different pH values to react with captured antibodies was assessed under continuous flow (5 μL/min). Each Mabs diluted at 1/100 in HEPES-buffered saline (HBS) was captured by a 25-min injection at a flow rate of 30 μL/min, which ensured immobilization of approximately 2000 resonance units (RU) of the Mabs on the RAM. One thousand RU correspond to an angle change of 0.1° and a protein concentration change of 1 ng/mm^2 (Fagerstam et al., 1990). In a sensorgram, the response signal expressed in RU is plotted as a function of time. The interaction between immobilized Mabs and antigens passing over the surface in a continuous flow is illustrated in the sensorgram represented in Figure 1.

RESULTS AND DISCUSSION

Selection of Specific Hybridoma. In the present study, we produced and characterized specific Mabs that

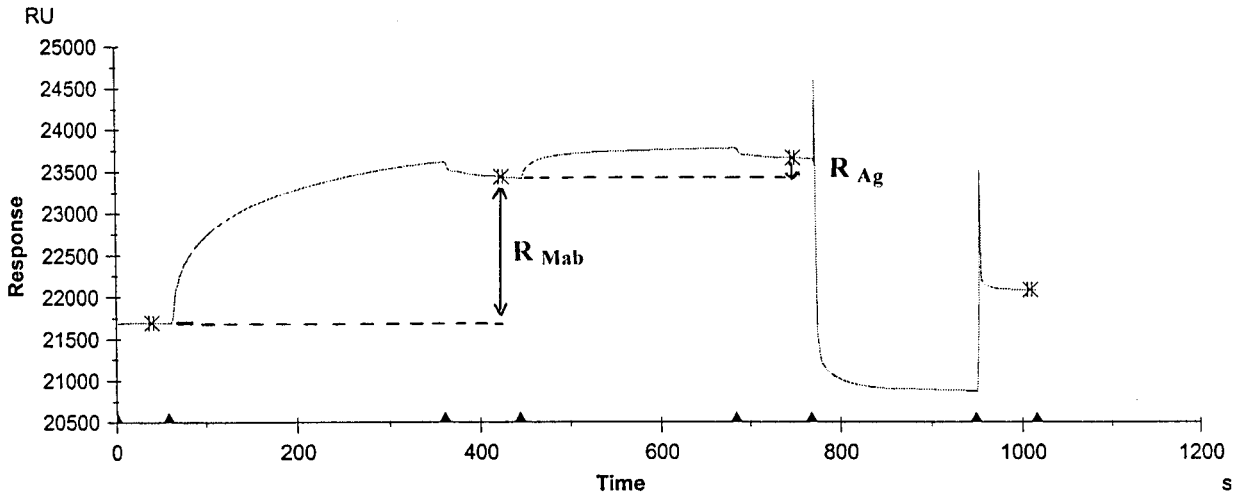

Figure 1. Typical sensorgram showing the interaction between a Mab and its antigen. The Mab is immobilized to the sensor surface by a rabbit anti-mouse immunoglobulin Fc (RAM); the following sequential injections were performed, and the RAM biocaptor was then regenerated with two pulses of 1 M formic acid. R_{Mab}, response of Mab immobilization; R_{Ag}, response of antigen binding to the immobilized Mab.

Table 1. Specificity of the Monoclonal Antibodies As Assessed by both Indirect and Sandwich ELISA[a]

	indirect ELISA				sandwich ELISA			
	104	139	918	444	104	139	918	444
porcine pepsin	++	---	+++	---	+++	+++	---	---
bovine pepsin	---	+++	+++	++	---	---	+++	++
chymosin	---	---	---	---	---	---	---	---
C. parasitica	---	---	---	---	---	---	---	---
M. pusillus	---	---	---	---	---	---	---	---

[a] Mabs reactivities were tested toward aspartyl proteases from both animal (porcine and bovine pepsin, chymosin) and fungal (*Crynophectria parasitica, Mucor pusillus*) sources. Experimental details are given in Materials and Methods.

could discriminate porcine pepsin from bovine pepsin and other milk coagulants. To obtain these Mabs, both porcine pepsin and purified bovine pepsin were used as antigens to produce monoclonal antibodies. Screening of these Mabs was first based on the ability of the hybridoma to produce immunoglobulins and then based on the ability of immunoglobulins to specifically recognize their immunogens. Among 3200 hybridoma produced, 365 were positive (results not shown) and were classified into three groups: highly, medium, and non specific according to their ability to recognize their immunogens on either unfolded (indirect ELISA) or native forms (sandwich ELISA). Most of the positive hybridoma interacted preferentially with antigens adsorbed on the plate. Changes in specificity of these Mabs seem to be highly dependent on the way antigens exhibited their determinants when adsorbed onto the solid phase and in solution, as shown in Table 1 for monoclonal antibodies 139 and 918. These results confirmed the influence of ELISA methods on Mabs specificity observed by several authors (Geysen, 1985; Friguet et al., 1984; Djavadi-Ohaniance et al., 1984; Vaidya et al., 1985).

The ability of our monoclonal antibodies to distinguish the structural homology among porcine pepsin, bovine pepsin, chymosin, and aspartyl proteases from either *Crynophectria parasitica* or *Mucor pusillus* was analyzed on both indirect and sandwich ELISA. Four hybridoma (104, 139, 918, and 444) from the highly specific group were retained as representatives of Mabs directed specifically against either porcine pepsin or bovine pepsin (Table 1). These Mabs interacted neither with chymosin nor with fungal aspartyl proteases. For further studies on their specificity toward native forms of porcine and bovine pepsins in solution, only 198 and 139 were used based on results from sandwich ELISA and preliminary BIA-core studies (results not shown). After twice limiting dilution of these two clones, one subclone was selected for each: 139(7) and 918(4). These Mabs were of the IgG1 isotype and were used later to produce ascitic fluids.

Characterization of Mabs Reactivities According to pH. Stability and specificity of antibody−antigen complex result from conformational complementarity between them. On the basis of the dogma that conformation dictates activity, we can suppose that changes in the structure of proteases could result in the loss of clotting activity as well as in either exposition or disappearance of some antigenic determinants.

To explore whether any of the obtained Mabs were able to recognize specifically either bovine or porcine pepsin showing conformational changes induced by fluctuations in pH during both cheese-making and ripening, ascitic fluids from 139(7) and 918(4) were analyzed on the BIA-core system. They were used as immunologic probes to explore the relationship between enzymatic activity and changes in antigenicity of pepsins according to pH. The range of pH used (from 5.0 to 8.0) was based on pH fluctuations in cheeses during both cheese-making and ripening, as described previously (Andren et al., 1983; Fox et al., 1969). The influence of pH on the clotting activities was demonstrated in this study (Figures 2 and 3). Moreover, loss of the clotting activity for porcine and bovine pepsins at pH 6.5 or at pH 7.5, respectively, was not reversible since the adjustment of pH back to the optimal pH did not result in the recovery of the enzyme activities (results not shown).

As also summarized in Figures 2 and 3, Mabs showed fluctuations in immunologic activities according to the antigen's pH. The sharp decrease in reactivity of 139(7) from 200 RU at pH 6.0 to 60 RU at pH 6.5 was parallel to the sharp decrease from 90% to 0% in residual clotting activity of porcine pepsin (Figure 2). These results indicate that an increase of 0.5 unit of pH between 6.0 and 6.5 is sufficient to affect both the immunologic reactivity of this Mab toward its antigenic determinants and the clotting activity of the enzyme.

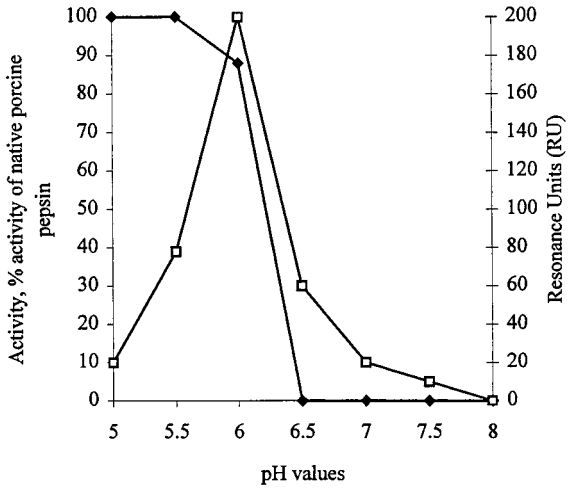

Figure 2. Influence of pH on enzymatic activity (% of residual clotting activity: (—◆—) and changes in antigenicity (resonance units: —) of porcine pepsin. Mab 139(7) was used as the immunological probe. Experimental details are given in Materials and Methods.

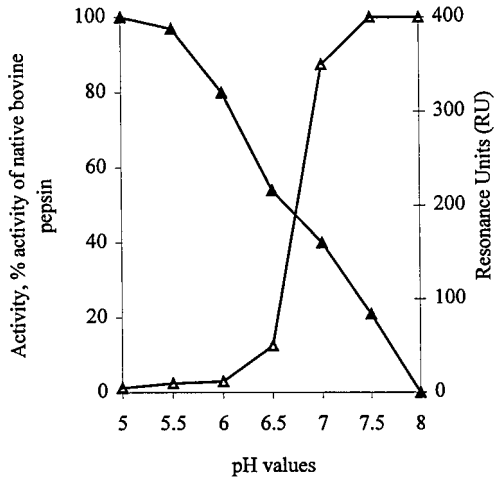

Figure 3. Influence of pH on enzymatic activity (% of residual clotting activity: —▲—) and changes in antigenicity (resonance units: —△—) of bovine pepsin. Mab 918(4) was used as the immunological probe. Experimental details are given in Materials and Methods.

Our results are consistent with those of Smith and Yada (1991). They demonstrated that an increase in pH by 0.5 from the optimal pH was sufficient to induce a conformational change in porcine pepsin. Our results speak strongly about the effect of pH on the accessibility of antigenic determinants and on the stability of the antigen−antibody complex. Thus, Mab 139(7) seems to be a powerful probe directed against a specific antigenic determinant whose maximal accessibility depends on the pH of the medium where porcine pepsin is in the native form.

A markedly different relationship was observed between the clotting activity and the immunological response of bovine pepsin. With increasing pH values, the decrease in clotting activity was accompanied by an increase in the immunological reactivity of 918(4) (Figure 3). At pH 8, the immunological activity was the highest (400 RU), while no residual clotting activity was observed. The denatured bovine pepsin did not have the immunological identity that can be recognized by Mab 918(4), when the enzyme was active at pH below 6.5. Mab 918(4) seems to be a powerful antigenic probe directed against a specific antigenic determinant whose accessibility reaches its maximal level when the bovine pepsin is inactivated at pH above 7 (no residual clotting activity).

Several studies have demonstrated that monoclonal antibodies with thoroughly characterized target specificities can be used as powerful probes of protein conformation (Fedorov et al., 1992; Larvor et al., 1991). In addition to providing information on the relative arrangement of the domains in both the native and denatured molecules, they can also be used to monitor both early and late stages of protein conformational changes (Larvor et al., 1991). By using the BIA-core system, we have demonstrated that monoclonal antibodies, through changes in their affinity toward their antigens, are powerful probes to study pH-induced conformational changes of both porcine and bovine pepsins. These results demonstrate not only that changes in the antigen binding site affect antibody binding but, more importantly, that changes in the antigenic determinants induced by local perturbations in structure can be detected by monoclonal antibody binding. These findings emphasize the role of conformation in the stabilization of the interaction between protein antigens and high-affinity monoclonal antibodies. Furthermore, monoclonal antibodies in our study are more specific and sensitive probes for detecting changes in conformation brought by pH than antisera as reported previously by Andren et al. (1983). These findings confirm that monoclonal antibodies can play an important role in detecting conformational changes caused by pH fluctuations.

In summary, this study revealed that monoclonal probes can be used in determining pH-induced changes in conformation of porcine and bovine pepsins; subsequently, these changes affect both their antigenicity and catalytic activities. Results obtained herein illustrate the advantage of Mabs screening according to their selectivity and stable nature of antigenic determinants, particularly when the native conformation of antigens is highly dependent on medium conditions, particularly pH. The selection of Mabs on the basis of their binding properties to antigens at different pH values represents an initial step in attempting to develop an enzyme-linked immunosorbent assay to detect and quantify active residual porcine pepsin in different cheeses.

LITERATURE CITED

Adad Zapatero, C.; Rydel, T. K.; Tang, J. Revised 2.3 Å structure of pepsin: evidence for a flexible subdomain. *Proteins* **1990**, *8*, 62−81.

Andren, A.; Dekoning, P. J.; Bjork, L. Changes in immunological and catalytically active site of chymosin, bovine and porcine pepsin. *Neth. Milk Dairy J.* **1983**, *37*, 11−20.

Berridge, N. J. Some obserevations on the determination of the activity of rennet. *Analyst* **1952**, 77, 57−62.

Boudjellab, N.; Grosclaude, J.; Zhao, X.; Collin, J. C. Development of an inhibition Enzyme Immunosorbent Assay for the detection of residual porcine pepsin in a soft cheese sample. *J. Agric. Food Chem.* **1998**, *46*, 4030−4033.

Collawn, J. F.; Wallace, C. J. A.; Proodfoot, A. E. I.; Paterson, Y. Monoclonal antibodies as probes of conformational changes in protein-engineered cytochrome C. *J. Biol. Chem.* **1988**, *263*, 8625−8634.

Collin, J. C.; Grapin, R.; Legreat, Y. Etude du temps de coagulation d'une solution enzymatique. *Rev. Laitiere Fr.* **1977**, *3555*, 1−5.

Collin, J. C.; Martin, P.; Garnot, P.; Ribadeau-Dumas, B.; Mocquot, G. Determination of chymosin and bovine A in

commercial bovine rennets and pepsins. *Milchwissenchaft* **1981**, *36*, 32−35.

De Blas, A. L.; Ratnaparki, M. V.; Mosimann, J. E. Estimation of the number of monoclonal hybridoma survival. *J. Immunol. Methods* **1981**, *45*, 109−115.

Djavadi-Ohaniance, L.; Friguet, B.; Goldberg, M. E. Structural and functional influence of enzyme-antibody interactions: effect of eight different monoclonal antibodies of *E. coli* tryptophan synthetase. *Biochemistry* **1984**, *23*, 97−104.

Fagerstam, M. G.; Frostell, A.; Karlsson, R.; Larssons, A.; Malmquist, A.; Butt, H. Detection of antigen−antibody interactions by surface plasmon resonance: Application to epitope mapping. *J. Mol. Recognit.* **1990**, *3*, 208−214.

Fedorov, A. N.; Friguet, B.; Djavadi-Ohaniance, L.; Alakhov, Y. B.; Godberg, M. E. Folding on the ribosome of *Esherichia coli* tryptophan synthase beta subunit nascent chains probed with a conformation-dependent monoclonal antibody. *J. Mol. Biol.* **1992**, *228*, 351−358.

Foltmann, B.; Pedreson, V. B. Comparison of the primary structures of acidic proteases and their zymogens. *Adv. Exp. Med. Biol.* **1977**, *95*, 3−22.

Foltmann, B. Gastric proteases-structure, function, evolution and mechanism of action. *Assays Biochem.* **1981**, *17*, 52−84.

Fox, P. F. Milk-clotting and proteolytic activities of rennet and of bovine pepsin and porcine pepsin. *J. Dairy Res.* **1969**, *36*, 427−433.

Friguet, B.; Djavadi-Ohaniance, L.; Goldberg, M. E. Some monoclonal antibodies raised with native protein bind preferentially to the denatured antigen. *Mol. Immunol.* **1984**, *21*, 673−677.

Fruton, J. S. The mechanism of the catalytic action of pepsin and related acid proteases. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1976**, *44*, 4411−4436.

Geysen, H. M. Antigen−antibody interactions at the molecular level: adventures in peptide synthesis. *Immunol. Today* **1985**, *6*, 364−369.

Godinot, C.; Moradi-Ameli, M.; Gautheron, D. C. Monoclonal antibodies to F_1-ATPase subunits as probes of structure conformation and functions of isolated membrane-bound F_1. *Methods Enzymol.* **1986**, *126*, 761−769.

Goldberg, M. E. Investigating protein conformation, dynamics and folding with monoclonal antibodies. *Trends Biochem. Sci.* **1991**, *16*, 358−362.

Guinee, T. P.; Wilkinson, M. G. Rennet coagulation and coagulants in cheese manufacture. *J. Soc. Dairy Technol.* **1992**, *45*, 94−104.

Hsu, I. N.; Delbaere, L. T. J.; James, N. G. Penicillopepsin from *penicillium janthinellum* crystal structure at 2.8 Å and sequence homology with porcine pepsin. *Nature* **1977**, *266*, 140−145.

Karlsson, R.; Michaelsson, A.; Mattson, L. Kinetic analysis of monoclonal antibody−antigen interaction with a new biosensor analytic system. *J. Immunol. Methods* **1991**, *145*, 229−240.

Katoh, J.; Yasunga, T.; Ikawa, Y.; Yoshinaka, Y. Inhibition of retroviral protease activity by an aspartyl protease inhibitor. *Nature* **1987**, *329*, 654−656.

Larvor, M. P.; Djavadi-Ohaniance, L.; Friguet, B.; Baleux, F.; Goldberg, M. E. Peptide/antibody recognition: synthetic peptides derived from the *E. coli* tryptophan synthase beta 2 subunit interact with high affinity with an anti-beta 2 monoclonal antibody. *Mol. Immunol.* **1991**, *28*, 523−531.

Lin, Y.; Fused, M.; Lin, X.; Hartsuck, J. A.; Tang, J. pH dependence of kinetic parameters of pepsin, Rhizopuspepsin and their active-site hydrogen bonds mutants. *J. Biol. Chem.* **1992**, *267*, 18413−18418.

Nelson, J. E. Symposium: application of enzyme technology to dairy manufacture of new enzymes on cheese technology. *J. Dairy Sci.* **1975**, *58*, 1739−1750.

Nowinsky, R. C.; Lonstom, M. E.; Tam, M. R.; Stone, M. R.; Burnette, W. R. The isolation of hybrid cell lines producing monoclonal antibodies against P15 (E) protein of electropic leukemia viruses. *Virology* **1979**, *93*, 111−126.

Phelan, J. A.; Guiney, J.; Fox, P. F. Proteolysis of B-casein in cheddar cheese. *J. Dairy* Res. **1973**, *40*, 105−112.

Schwab, C.; Bosshard, H. R. Caveats for the use of surface adsorbed protein antigen to test the specificity of antibodies. *J. Immunol. Methods* **1992**, *147*, 125−134.

Shulman, M.; Wide, C. D.; Kohler, G. A better cell line for making hybridomas secreting specific antibodies. *Nature* **1978**, *176*, 269−270.

Sielecki, A. R.; Fedorov, A. A.; Boodho, A.; Andreeva, N. S.; James M. N. G. The molecular and crystal structures of monoclonic porcine pepsin refined at 1.8 Å resolution. *J. Mol. Biol.* **1990**, *214*, 143−170.

Smith J. L.; Yada, R. Y. Chemical modification of amino groups in *Mucor miehei* aspartyl protease, porcine pepsin and chymosin: II-conformational stability. *Agric. Biol. Chem.* **1991**, *55*, 2017−2024.

Tang, J. Sepulveda, P.; Marcinszyn, J., Jr.; Chen, K. C. S.; Huang, W. Y.; Tao, N.; Liu, D.; Lanier, J. P. Amino-acid sequence of porcine pepsin. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 3429−3437.

Umezawa, H.; Aoyagi, T.; Morishima, H.; Matsuzaki, M.; Hamada, M.; Takeuchi, T. A new inhibitor produced by actinomycetes. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *83*, 4147−4151.

Vaidya, H. C.; Dietzler, D. N.; Laderson, J. H. Inadequacy of traditional ELISA for screening hybridoma supernatants for murine monoclonal antibobies. *Hybridoma* **1985**, *4*, 271−276.

Van Regenmortel, M. H. V. Structural and functional approaches to the study of protein antigenicity. *Immunol. Today* **1989**, *10*, 266−272.

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