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Detection of recombinant chymosins in calf rennet by enzyme-linked immunosorbent assay

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Summary — The presence and the origin of recombinant chymosin can be detected in milk-clotting enzyme solutions by antigen coat plate-enzyme-linked immunosorbent assay (ACP-ELISA) or sandwich ELISA. The method is based on the detection of contaminant proteins derived from microorganisms or from the culture medium, which are always present in fermentation-produced chymosin solutions. The specific polyclonal antibodies obtained from the sera of rabbit and hen permitted identification of the contaminant proteins in the commercial solutions. A simple dilution of the sample is sufficient to detect the addition of 0.5% Chymogen (Hansen) or Maxiren (Gist-Brocades) and 2.5% Chymax (Pfizer) in calf rennet.

recombinant chymosin / detection / identification / ELISA / calf rennet

Résumé — **Détection des chymosines d'origine fermentaire dans la présure par technique immunochimique ELISA.** Des méthodes immunochimiques ACP-ELISA et ELISA sandwich permettent de détecter la présence et l'origine de chymosine produite par génie génétique dans une solution d'enzyme coagulante. Cette détection se fait par l'intermédiaire des protéines contaminantes provenant du microorganisme hôte ou du milieu de culture, toujours présentes dans les solutions de chymosine fermentaire. Les antisérums spécifiques obtenus chez le lapin et la poule permettent d'identifier les protéines contaminantes présentes dans les préparations de chymosines recombinantes. Par une simple dilution de l'échantillon, il est possible de détecter l'ajout de 0,5 % de Chymogen (Hansen) ou Maxiren (Gist-Brocades) et de 2,5 % de Chymax (Pfizer) dans une présure.

chymosine fermentaire / détection / identification / ELISA / présure

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INTRODUCTION

The increase in world cheese production has led to widespread utilisation of milk clotting agents. As rennet extracted from the stomach of calves could no longer meet world demands, rennet substitutes such as bovine pepsin, porcine and chicken pepsin and microbial protease produced by *Mucor miehei*, *Mucor pusillus* and *Cryphonectria parasitica* have been used for many years.

These rennet substitutes, like chymosin, are acid proteases. Pepsins, having a marked proteolytic activity, are responsible for bad flavours in cheese (Van den Berg, 1992). It has been found that cheese yields obtained from fungal enzymes are not as good as those from chymosin (Emmons and Binns, 1991). Chymosin is therefore considered to be the best clotting enzyme for cheese production.

Since the 1980s, genetic engineering techniques have been used to produce chymosin (Teuber, 1990; Beppu, 1991; Yu, 1994). Today three different companies produce recombinant chymosin from three different micro-organisms. The companies, ie, Chr Hansen (Denmark), Gist-Brocades (France) and Pfizer (USA) produce Chymogen from the fungus *Aspergillus niger* var *awamori* (Halfhide, 1992; Harboe, 1992), Maxiren from the yeast *Kluyveromyces lactis* (Praaning-Van Dalen, 1992; Leeuw and Swinkels, 1993), and Chymax from the bacterium *Escherichia coli* K12 (Lynch, 1994), respectively.

The marketing of these recombinant chymosins is permitted in many countries, for example in Australia, Belgium, Great Britain, Greece, Israel, Portugal, Switzerland and the USA (Brusgaard, 1992).

The analytical identification of these different chymosins is essential for several reasons. For ethical reasons, it is always preferable to know the additives used for manufacture of food products when possible. AOP cheeses (Appellation d'Origine Pro-

tégée) must be produced with animal rennet: therefore an analytical technique which differentiates between the commercial solutions is necessary. Finally, it is important to be able to detect whether or not recombinant chymosin has been added to bovine pepsin as a method to artificially reconstitute rennet.

Chymosin produced by genetically engineered microorganisms is identical in molecular weight, amino-acid sequence, immunological properties and chromatographic profile to natural chymosin (Corradini et al, 1993). Numerous studies have shown that in cheese production the yield and quality of the finished product is the same whatever the origin of the chymosin (Bines et al, 1989; Prokopek et al, 1990; Van den Berg and de Koning, 1990). All commercial solutions of recombinant chymosin contain small quantities of contaminant proteins due to producer microorganisms or to the culture medium. These impurities are always present in solutions even after purification. Recombinant chymosins will therefore be detected through their contaminant or secondary proteins.

Panari and Corradini (1993) have suggested differentiating between animal and recombinant chymosins by HPLC (high performance liquid chromatography).

Our work included the preparation of antibodies against secondary proteins of recombinant chymosin solutions and also the development of enzyme-linked immunosorbent assay (ELISA) techniques so that these proteins, present when chymosin of genetic origin has been added to a clotting enzyme solution, could be detected and recognised.

MATERIALS AND METHODS

Antigens

The antigens were purified by the three recombinant chymosin-producing firms according to

their respective purification protocols. The antigen solutions had to be free from chymosin molecules.

The antigen provided by Hansen (Chr Hansen's Laboratorium, 2970 Hørsholm, Denmark) came from the fermentation of the genetically manipulated *A niger* var *awamori* with the chymosin gene deleted. Secondary contaminant proteins were isolated by FPLC (fast protein liquid chromatography) (Pharmacia Biotech AB, Uppsala, Sweden) on phenyl-Sephadex. These proteins, eluted before chymosin, were desalted on a PD-10 column (Pharmacia), freeze-dried, dissolved in physiological salt water (0.9% NaCl), dialysed against a solution of 0.9% NaCl and finally frozen. The protein concentration was determined by the method of Lowry (75 mg/mL).

The antigen from Gist-Brocades (Gist-Brocades, Seclin, France) was prepared by fermentation of the strain *K lactis* with the deletion of the gene coding for chymosin. The culture conditions and treatment were the same as for normal chymosin fermentation. The supernatant was concentrated on an ultrafiltration membrane with a molecular weight cut-off of 100 000 Da. The protein concentration was determined by the method of Bradford (3.7 mg/mL).

The antigen from Pfizer (Pfizer, Milwaukee, WI, USA) was prepared by fermentation of the strain *E coli* K12, which did not contain the cDNA encoding for prochymosin. The absence of chymosin in the antigen solution was tested by milk-clotting activity assay, by anion exchange HPLC and gel permeation chromatography with diode array detection. The protein concentration was determined by BCA protein assay (0.09 mg/mg) (Pierce, Rockford, IL, USA).

Production of polyclonal antibodies against recombinant chymosin

Polyclonal antibody production by rabbit

Commonbred rabbits were immunised with Hansen, Gist-Brocades or Pfizer antigens. Each rabbit was injected subcutaneously with 0.5 mg of antigen emulsified in Freund's complete adjuvant (DIFCO Laboratories, Detroit, MI, USA) in multiple sites. The first booster injection was given 21 days after the first immunisation. The antigen (0.5 mg) was emulsified in Freund's incomplete adjuvant. A second booster injection

was given 15 days later. The rabbits were bled 10 days after the last booster injection. After clotting at 4 °C, the blood was centrifuged at 1800 g for 5 min (J2-21 M/E Beckman Instruments Inc Palo Alto, CA, USA). The three sera containing the antibodies against Hansen, Gist-Brocades or Pfizer antigens were collected and dialysed in an ammonium hydrogeno-carbonate buffer 0.01 mol/L, pH 7.0, tested by ELISA and then stored at -18 °C.

Polyclonal antibody production by chicken

Only the antibodies against the Pfizer antigen were synthesised in the chicken. A commonbred chicken was given 0.3 mg antigen emulsified in Freund's complete adjuvant in an intramuscular injection. A booster of 0.3 mg antigen emulsified in Freund's incomplete adjuvant was given 21 days later. The eggs were collected daily 10 days after the booster injection and separated into groups corresponding to a 10-day laying period. The immunoglobulins were extracted from the yolk according to the technique described by Polson et al (1980). Finally they were dialysed in an ammonium hydrogeno-carbonate buffer 0.01 mol/L, pH 7.0 and freeze-dried.

Methods for recombinant chymosin detection

ACP-ELISA (antigen coat plate)

The ACP-ELISA method was used for the detection of both Hansen (*A niger*) and Gist-Brocades (*K lactis*) antigens.

The solution to be analysed was diluted 10-fold in a carbonate buffer 0.1 mol/L, pH 9.6. A flat-bottomed polystyrene microplate (Maxisorp; Nunc, Roskilde, Denmark) was coated with 100 µL solution per well. The plate was then incubated overnight at 4 °C or for 90 min at 37 °C. After incubation, it was washed four times with 150 µL of 0.05 mol/L sodium phosphate-0.15 mol/L NaCl buffer, pH 7.2 containing 0.5 mL Tween 20/L (PBST). Between each subsequent step, the plate was washed four times with PBST. The plate was saturated with 200 µL per well of a 3% gelatine solution dissolved in PBST (w/v) (Merck, Darmstadt, Germany), and

incubated for 60 min at 37 °C. One hundred microliters rabbit antibodies (anti-Chymogen or anti-Maxiren) diluted in PBST were added to each well, and the plate was incubated for 90 min at 37 °C; 100 µL goat anti-rabbit immunoglobulin-alkaline phosphatase conjugate (Sigma GAR-PAL, goat anti-rabbit-alkaline phosphatase) (Sigma Chemical Co, St Louis, MO, USA) diluted in PBST were added to each well. The plate was incubated for 90 min at 37 °C. The activity of the alkaline phosphatase of the conjugate bound to the antibodies was detected by the addition of 100 µL *p*NPP (*para*-nitrophenyl-phosphate) at 1 mg/mL in 0.1 mol/L diethanolamine buffer, pH 9.8. The plate was incubated for 1 h at 37 °C, and the absorbance of each well measured at 405 nm using an Anthos Labtec HT3 spectrophotometer (Anthos Labtec Instruments, Salzburg, Germany). The dilutions of antiserum and conjugate were chosen to obtain an optical density (OD) (1.5 for the positive sample after 1 h at 37 °C. They were 1/200 and 1/5000, respectively.

The ACP-ELISA method was also used to test the specificity of rabbit with regard to their respective antigens and the lack of reaction with chymosin. The specificity of chicken immunoglobulins was tested by ACP-ELISA. The conjugate used was an anti-chicken immunoglobulin-peroxidase conjugate (Sigma RACH-PO, rabbit anti-chicken-peroxidase). The activity of the peroxidase was detected by the addition of 75 µL substrate (0.3 mg, 3,3', 5,5'-tetramethylbenzidine/mL of 1 mol/L citrate buffer pH 5.0 containing 4 g H₂O₂/L). After incubation for 15 min at 37 °C, the reaction was stopped with 25 µL 1 mol/L HCl / well. The absorbance was measured at 405 nm.

ELISA sandwich or DAS-ELISA (double sandwich antibodies)

The ELISA sandwich method was used to detect the Pfizer antigen (*E coli* K12). As was carried out in the ACP-ELISA, the plate was washed four times with PBST between each subsequent step. The plate was coated with 100 µL chicken antibodies IgY (capture antibody) at a concentration of 10 µg freeze-dried IgY/mL in a 0.1 mol/L carbonate buffer, pH 9.6, then incubated for 90 min at 37 °C or overnight at 4 °C. As in the ACP-ELISA, the plate was saturated with 200 µL per well of a 3% gelatine solution dissolved in PBST and then incubated for 60 min at 37 °C. The sample containing the antigens was

diluted 50% in PBST, and 100 µL of this solution were added to the coated well. The subsequent steps, ie, the addition of rabbit antibodies (anti-Chymax), anti-rabbit immunoglobulin-alkaline phosphatase conjugate and *p*NPP substrate were identical to the ACP-ELISA technique. The dilutions of rabbit antibodies and conjugate were chosen so as to obtain an OD \geq 1.5 for the positive sample after 1 h at 37 °C. They were 1/100 and 1/2000, respectively.

RESULTS

Detection of Chymogen (*A niger*) in rennet

The results obtained in ACP-ELISA with anti-Chymogen rabbit antiserum are shown in figure 1. The solutions of Chymogen, Maxiren and Chymax were diluted 20, 10

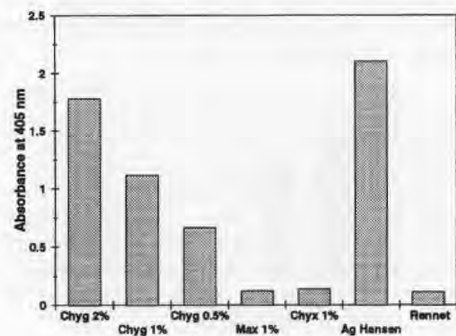


Fig 1. Detection of the Hansen antigen (Chymogen) by ACP-ELISA. The solutions Chymogen (Chyg), Maxiren (Max) and Chymax (Chyx) were diluted 20, 10 and 5% in rennet, and then 1/10 in 0.1 mol/L carbonate buffer pH 9.6. Ag Hansen: Hansen antigen, 5 ng/mL. Rennet: rennet at 520 mg chymosin / L diluted 1/10. Anti-Chymogen 1/200, conjugate GAR-PAL 1/5000. *Détection de l'antigène Hansen (Chymogen) par ACP-ELISA. Les solutions de Chymogen (Chyg), Maxiren (Max) et Chymax (Chyx) sont diluées à 20, 10 et 5 % dans la présure, puis au 1/10 dans 0,1 mol/L de tampon carbonate pH 9,6. Ag Hansen : antigène Hansen, 5 ng/mL. Rennet : présure à 520 mg chymosine / L diluée au 1/10. Anti-Chymogen 1/200, conjugué GAR-PAL 1/5000.*

and 5% in a commercial rennet solution containing 520 mg active bovine chymosin / L.

The ACP-ELISA method allowed recombinant chymosin added to a clotting solution to be identified. Maxiren and Chymax solutions, as well as rennet, did not show any cross-reaction with the antibody ($OD < 0.2$). Only Chymogen solutions diluted 20, 10 and 5% in rennet and the solution containing the antigen used for the production of the anti-Chymogen antibodies were recognised ($OD > 0.6$). The anti-Chymogen antiserum was very specific.

Detection of Maxiren (*K lactis*) in rennet

The results obtained in ACP-ELISA with anti-Maxiren rabbit antiserum are shown in figure 2. The Chymogen, Maxiren and Chy-

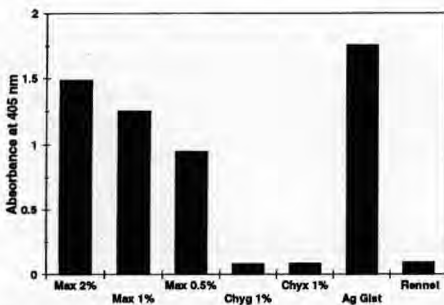


Fig 2. Detection of the Gist-Brocades antigen (Maxiren) by ACP-ELISA. The solutions Chymogen (Chyg), Maxiren (Max) and Chymax (Chyx) were diluted 20, 10 and 5% in rennet, and then 1/10 in 0.1 mol/L carbonate buffer pH 9.6. Ag Gist: Gist-Brocades antigen, 5 ng/mL. Rennet: rennet at 520 mg chymosin / L diluted 1/10. Anti-Maxiren 1/200, GAR-PAL conjugate 1/5000. *Détection de l'antigène Gist-Brocades (Maxiren) par ACP-ELISA. Les solutions de Chymogen (Chyg), Maxiren (Max) et Chymax (Chyx) sont diluées à 20, 10 et 5 % dans la présure, puis au 1/10 dans 0,1 mol/L de tampon carbonate pH 9,6. Ag Gist : antigène Gist-Brocades, 5 ng/mL. Rennet : présure à 520 mg chymosine / L diluée au 1/10. Anti-Maxiren 1/200, conjugué GAR-PAL 1/5000.*

max solutions were diluted 20, 10 and 5% in the rennet, as previously described.

Regarding the detection of Chymogen, the ACP-ELISA method was retained to identify Maxiren. The Maxiren solutions diluted in rennet 20, 10 and 5% and the antigen solution used for the production of the anti-Maxiren antibodies were the only antigens to show a positive reaction ($OD > 0.9$). Neither Chymogen and Chymax solutions nor rennet reacted with the anti-Maxiren antiserum ($OD < 0.1$).

Detection of Chymax (*E coli*) in rennet

Owing to its specificity and sensitivity, the ELISA sandwich method was chosen to detect the presence of Chymax in rennet. The results obtained with anti-Chymax rabbit antiserum are presented in figure 3. Chy-

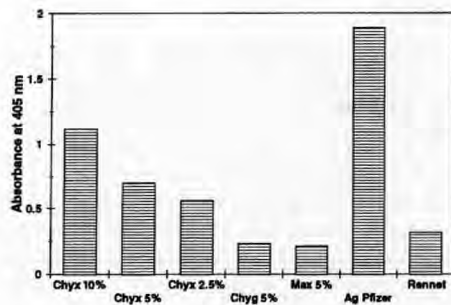


Fig 3. Detection of the Pfizer antigen (Chymax) by ELISA sandwich. The solutions Chymogen (Chyg), Maxiren (Max) and Chymax (Chyx) were diluted 20, 10 and 5% in rennet, and then 1/2 in 0.1 mol/L carbonate buffer pH 9.6. Ag Pfizer: Pfizer antigen, 25 ng/mL. Rennet: rennet at 520 mg chymosin / L diluted 1/2. IgY 10 µg/mL, anti-Chymax 1/100, GAR-PAL conjugate 1/2000. *Détection de l'antigène Pfizer (Chymax) par ELISA sandwich. Les solutions de Chymogen (Chyg), Maxiren (Max) et Chymax (Chyx) sont diluées à 20, 10 et 5 % dans la présure, puis au 1/2 dans 0,1 mol/L de tampon carbonate pH 9,6. Ag Pfizer : antigène Pfizer, 25 ng/mL. Rennet : présure à 520 mg chymosine / L diluée au 1/2. IgY 10 µg/mL, anti-Chymax 1/100, conjugué GAR-PAL 1/2000.*

mogen, Maxiren and Chymax solutions were diluted 20, 10 and 5% in rennet.

Only the solutions with 20, 10 and 5% Chymax and the Pfizer antigen used to produce the anti-Chymax antibodies were recognised by these antibodies ($OD > 0.55$). The absorbance of Chymogen and Maxiren solutions and rennet was weak ($OD < 0.32$).

DISCUSSION AND CONCLUSION

The ELISA techniques developed in this study allow the presence and origin of recombinant chymosins added to clotting solutions to be detected. These techniques are simple because they only require dilution of the sample before the assay.

The ACP-ELISA method allows detection of the addition of 0.5% Chymogen or Maxiren to a commercial clotting enzyme solution.

Since contaminant proteins were present in low concentration in Chymax solutions, the ACP-ELISA was not sufficiently sensitive. Owing to the widespread presence of *E coli* in the environment, antigens of this microorganism may have been present in small quantities in the other clotting enzyme solutions, which could explain the significant background noted ($OD > 0.35$).

As the ELISA sandwich method raises the sensitivity of the reaction, it is possible to detect the addition of 2.5% Chymax in a commercial solution of clotting enzyme. However, it is difficult to interpret the results for an addition of Chymax of under 2.5%, because the background response remains high.

The specificity of each antigen to each strain permits the differentiation between and identification of recombinant chymosin solutions. The sensitivity of the ELISA techniques permits the identification of the impurities that are always – even in minute quantities – present in commercial solutions.

The ELISA methods described do not allow recombinant chymosin in cheese to be identified. Indeed, during cheese-making, most of the clotting solution is carried away in whey. The quantity of contaminant proteins in curd is very small and below the sensitivity level of the method. In addition, *K lactis* and *E coli* antigens can be naturally present in milk and cheese and induce misleading reactions.

The techniques described in this article may provide an efficient method of determining the continuity of the procedure used for enzyme production. The production and commercialisation of an enzyme are authorised by the competent ministry once a precise technical file and well-defined operation method have been supplied. If a change in strain, culture medium or operational method occurs it will bring about a change in the nature or concentration of the antigens.

In the identification and control of new products obtained by genetic engineering, the determination of contaminant proteins by ELISA techniques presents many possibilities for future development.

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