Development of an Inhibition Enzyme-Linked Immunosorbent Assay for the Detection of Residual Porcine Pepsin in a Soft Cheese Sample

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An inhibitory ELISA assay was developed for the detection of residual porcine pepsin in a fresh soft cheese. The concentration of porcine pepsin required to inhibit 50% of the binding of the antiserum in this ELISA assay was ∼81 ng/mL. The limit of detection of the ELISA method was ∼10 ng/mL, and the coefficient of variation was <10%. This method uses an antiserum capable of dicscriminating porcine pepsin from chymosin and proteinase from *Cryphonectria parasitica* but not from bovine pepsin. The adsorption of the antiserum against bovine pepsin resulted in a decrease of its cross-reactivity with this milk coagulant. The selected pH of trisodium citrate solution (pH 6) for the dissociation of curd caseins and for the assay was compatible with ELISA and resulted in acceptable recoveries of residual porcine pepsin in a fresh soft cheese sample.

Keywords: *Porcine pepsin; ELISA; polyclonal antibody; pH; cheese*

INTRODUCTION

Porcine pepsin has been widely used in cheese-making processes; it has been used in Europe and elsewhere since the 1960s (Crawford, 1988; IDF, 1990; Guinee and Wilkinson, 1992). Porcine pepsin−rennet blends were estimated to account for the major portion of cheese production in the United States (Nelson, 1975). The quality and taste of cheeses depend largely on the presence and activity of residual proteinases. These enzymes vary in their activities and specificities, which affect the rate and type of proteolysis and thus the development of texture and flavor. It is therefore imperative that the cheese-maker has a clear understanding of residual proteinases' function during both the cheese-making and ripening processes. In addition, a specific and sensitive method for reliable detection of porcine pepsin in cheeses is warranted for both religious and regulatory requirements. Whereas rennet has been intensively studied, fewer studies have been carried out for the identification and quantification of residual porcine pepsin. Attempts to measure the level of porcine pepsin in curd by extraction methods do not guarantee complete recovery of the residual enzyme. Holmes and Ernstrom (1973) were unable to recover any milk coagulant enzyme from cheddar cheese curd manufactured with porcine pepsin. They attributed this to the extensive denaturation of the labile enzyme and its adsorption by caseins. Additionally, only 5% of the added calf rennet were detected. Thomasow (1971) demonstrated that porcine pepsin was destroyed during the cheese-making process of Cheddar cheese and that the majority of this enzyme might be lost in the whey. Green (1972) suggested with reservations that all of the porcine pepsin added as coagulant during Cheddar cheese-making might be inactivated by the end of the process. On the other hand, others have demonstrated that porcine pepsin was considerably more stable in cheese than in liquid systems (O'Keefe et al., 1975). Furthermore, it was as stable as chymosin in cheeses (O'Keefe et al., 1977), and it maintained proteolytic activity for seven months in Cheddar cheese (Green and Foster, 1974). However, the current assays for determining either the presence or the activities of residual milk coagulants (Holmes and Ernstrom, 1973; Stadhouders et al., 1977; Matheson, 1981) are insensitive and generally need extraction, which is laborious and not suitable for routine analyses of cheese samples. All of the conflicting results in terms of the detection of residual porcine pepsin in cheeses show clearly the need for the development of a sensitive, specific, and simple method, which can be used as a rapid screening method for a large number of samples. Recently, immunoassays have become the most commonly used assays in food analysis. Among them, the enzyme-linked immunosorbent asssay (ELISA) is widely used for milk and dairy products. Its usefulness has been demonstrated for either the detection or the quantification of the residual activity of chymosin in cheeses (Taniguchi et al., 1987; Andersson et al., 1989; Boudjellab et al., 1994). ELISA methods were also used to detect substitution of goats' and ewes' milk by cows' milk (Levieux et al., 1993), proteolysis in raw milk stored at low temperatures (Picard et al., 1994), and quantitation of both plasmin and plasminogen in bovine milk samples (Dupont et al., 1997).

In this paper we describe an inhibition ELISA that uses a polyclonal antibody directed against porcine pepsin to detect and measure the presence of residual porcine pepsin in a fresh soft cheese. The reliability of this ELISA method was based on the pH of cheese

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samples during preparation and during ELISA assay. This method provides a high level of sensitivity, specificity, and reproducibility. Consequently, it is especially useful for detecting low amounts of both free and retained porcine pepsin in whey and curd samples, respectively.

MATERIALS AND METHODS

Materials. Crystalline porcine pepsin and gelatin were purchased from Merck (AG, Darmstadt, Germany); bovine pepsin and proteinase from *Cryphonectria parasitica* were purchased from Granday (Beaune, France). Porcine pepsin and proteinase from *C. parasitica* were of the highest grade commercially available and were used both for cheese-making and ELISA assays without further purification. Complete and incomplete Freund's adjuvant (CFA, IFA, respectively) were from Difco (Detroit, MI). Tween 20, *p*-nitrophenyl phosphate, bovine casein, diethanolamine, sulfate ammonium, and goat anti-rabbit IgG coupled to alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO). A Stomacher 400 lab-blender was obtained from Bioblock (Vanves, France). Microtiter plates were from Nunc (Roskilde, Denmark). All other solvents and reagents were of analytical quality.

Polyclonal Antibodies. Four male New Zealand white rabbits, ~3 months of age, were immunized at 2 week intervals during 4 months with crystalline porcine pepsin. The first injection consisted of 0.5 mg of the immunogen prepared in 0.5 mL of distilled water followed by the emulsification with an equal volume of CFA. The mixture was administered subcutaneously at multiple sites along the back of each animal as described previously (Vaitukaitis, 1981). Boosters consisted of 0.5 mg of the immunogen in 0.5 mL of distilled water and were emulsified with an equal volume of IFA. The boosters were given subcutaneously at multiple sites along the back. Animals were bled 8−10 days after each booster injection, and the blood was allowed to clot for 12 h at room temperature. Crude antiserum was collected by centrifugation at 2000*g* for 10 min and stored frozen at −20 °C. The Ig fractions were recovered from the crude antiserum by ammonium sulfate precipitation following the procedure previously described by Harlow and Lane (1988).

The specificity of the semipurified antiserum produced was assessed by the antigen coated on plate (ACP)-ELISA. Briefly, microplates were coated overnight at 4 °C with 100 μL of the antigens (bovine pepsin, chymosin, microbial proteinase from *C. parasitica*, and both native and heat-denatured porcine pepsin) at 5 μg/mL in 0.05 M sodium bicarbonate buffer, pH 9.6. Plates were then blocked at 37 °C for 1 h with 0.5% gelatin in PBST (w/v). Following this, 100 μL of a serial dilution of the semipurified antiserum was added and incubated for 1.5 h at 37 °C. After addition of 100 μL of adequately diluted anti-rabbit IgG−alkaline phosphatase conjugate diluted in PBST (1:2000), plates were incubated at 37 °C for 1 h. Subsequent steps were identical to those described for the inhibition ELISA (see below).

The adsorption of the antiserum Pp44 against bovine pepsin was carried out according to the method described by Avrameas and Ternynck (1969) to decrease its cross-reaction.

Preparation of Whey and Curd Samples. Soft cheese of the Camembert type was made using porcine pepsin as milk coagulant in the laboratory-automated mini-cheese pilot plant (vats of 10 L). As a negative control, the same type of cheese was made with milk coagulant from *C. parasitica*. Cheese samples were collected 20 h after cheese-making and whey samples at the end of drainage and pressing.

Cheese samples (5 g) were suspended in 25 mL of 0.4 M trisodium citrate solution (TCS pH 7 or 6) and mixed for 1 h to allow total dissolution of the caseins. Cheese suspension and the corresponding whey were then centrifuged for 15 min at 7000*g* at 4 °C. The cleared supernatant was recovered. Samples from the supernatant were divided and stored at −20 °C or directly analyzed in the inhibition ELISA. These samples were diluted in TCS adjusted to either pH 7 or 6 with 1 N HCl to obtain final dilutions of 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64 at the desired pH.

Table 1. Specificity of the Antiserum Pp44 before (B) and after (A) Adsorption against Bovine Pepsin As Determined by ACP-ELISA[a]

	% of cross-reactivity[b]	
proteinase	B	A
porcine pepsin	100	93.3
porcine pepsin[c]	83.5	66.6
bovine pepsin	86.6	34.5
chymosin	≤0.5	≤0.5
C. parasitica	≤0.5	≤0.5

[a] The antigens were adsorbed onto the solid phase at 5 μg/mL. [b] The cross-reactivity was determined as percentage of the maximal optical density obtained with porcine pepsin [% = (OD with bovine pepsin, porcine pepsin,[c] chymosin, or proteinase from *C. parasitica*/OD with porcine pepsin) × (100)]. [c] Heat-denatured porcine pepsin (10 min at 100 °C).

Inhibition ELISA. Flat-bottomed micro-ELISA microplates were coated at 4 °C overnight with 100 μL of pocine pepsin solution (10 μg/mL of 0.05 M sodium carbonate buffer, pH 9.6). The plates were then washed four times with 0.05 M phosphate, 0.15 M NaCl buffer, pH 7.4, containing 0.55 g of Tween 20/L (PBST), and subsequently blocked with 0.5% gelatin in PBST (w/v) for 1 h at 37 °C. Plates following blocking were washed three times with PBST. The anti-porcine pepsin serum Pp44 diluted at 1/2000 and the inhibitor (standards or samples), prepared in TCS (pH 6 or 7), were mixed in hemolysis tubes, and the immune reaction was allowed to take place for 1.5 h at 37 °C. A standard curve was obtained with purified porcine pepsin at final concentrations of 0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, and 2.5 μg/mL. After four washings with PBST, 100 μL of the antigen−antibody mixture was added to wells, and the plates were incubated at 37 °C for 1.5 h. Following three cycles of washing, 100 μL of goat anti-rabbit IgG−alkaline phosphatase conjugate diluted in PBST (1/2000) was added to all wells and allowed to incubate at 37 °C for 1 h. After three more washings, 100 μL of the substrate solution (1 mg of *p*-nitrophenyl phosphate disodium salt/mL, in 0.1 M diethanolamine buffer, pH 9.8) was added. The plates were allowed to develop for 1 h at 37 °C, and absorbances were determined on an ELISA plate reader (Anthos Labtec HT3, J.Bio, Les Ulis, France) fitted with a 405 nm filter.

RESULTS AND DISCUSSION

The antiserum is the most important ingredient in immunoassays. Its specificity and affinity determine not only the specificity and the sensitivity of the assay but also the practicability of methods. The characterization of the antiserum Pp44 was performed by the ACP-ELISA, which allowed us to select it on the basis of its specificity and sensitivity. As shown in Table 1, Pp44 interacted neither with chymosin nor with proteinase from *C. parasitica*. However, owing to the high structural similarity between porcine and bovine pepsins, no species-specific polyclonal antisera against porcine pepsin were produced in rabbits. The cross-reactivity observed with bovine pepsin was decreased by up to 60% by the adsorption of Pp44 against bovine pepsin. This cross-reaction with bovine pepsin can be avoided only by producing specific monoclonal antibodies. In addition, the antiserum produced with the native porcine pepsin shared a cross-reactivity with the same heat-denatured protein. This cross-reactivity suggests that the antiserum contains antibodies directed against epitopes present on both native and heat-denatured porcine pepsin. It has also been observed previously by

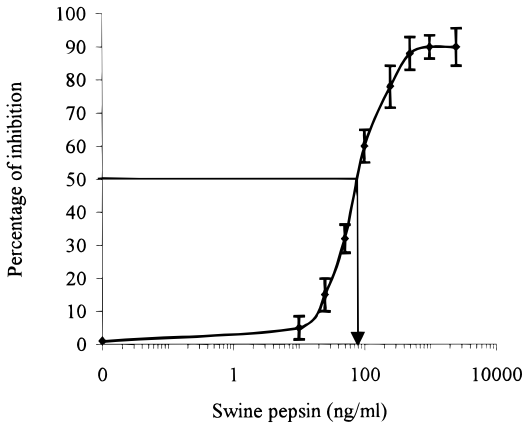

Figure 1. Typical inhibition ELISA standard curve for the detection of porcine pepsin. Values represent means ± SD. Experimental details are given in the text.

others that the antiserum produced with native protein cross-reacted with the same denatured protein (Crumpton, 1974; Friguet et al., 1984). Different ELISA procedures have been tested, and the inhibition ELISA was more consistent and sensitive than both the ACP and sandwich ELISA for our cheese extract samples (data not shown). An inhibition ELISA using the Pp44 to detect residual porcine pepsin in a fresh soft cheese and whey has been developed and reported here. As shown in Figure 1, the concentration of porcine pepsin required to inhibit 50% of the binding in the inhibition ELISA was ~81 ng/mL, with the lowest detectable concentration being ≤10 ng/mL, which corresponded to 10% inhibition. The repeatability of the method was estimated by analysis of variance of 10 replicates, and the coefficient of variation was <10%.

Using the established inhibition ELISA, residual porcine pepsin was identified in a whey sample from the soft cheese preparation. The whey sample from soft cheese preparation inhibited the immune reaction at a level of 59%. This percentage of inhibition corresponded to ~95.6 ng of residual porcine pepsin/mL, which represented ~80% of the total amount of porcine pepsin added to milk. The validity of the ELISA test (no inhibition: ≤10%) was also verified using the whey sample from the soft cheese manufactured with *C. parasitica.* These results demonstrated the specificity of the inhibition ELISA toward porcine pepsin and suggested that the antiserum contains antibodies directed against specific epitopes representing residual porcine pepsin. Unlike for whey samples, the detection of residual porcine pepsin in curd seems to be highly dependent on the pH of the TCS solution used both for sample preparation and during the ELISA test. As shown in Figure 2, both the negative control (curd of the soft cheese manufactured with *C. parasitica*) and the solution of whole bovine casein had very high false levels of inhibition when they were prepared at pH 7. Thus, it would be impossible to detect residual porcine pepsin in cheeses at such a pH value. In contrast, the same curd sample when prepared at pH 6 had acceptable results (≤12.5% of inhibition). These results indicated that the antiserum reacted with casein solutions at pH 7. The possible explanation for the discrepancy of the results at these two pH values is that the charges of caseins at pH 7 allow them to bind with immunoglobulines of Pp44 and then to interfere in the immune reaction. As shown in Figure 3, when the assay was performed at pH 6, no cross-reaction was observed

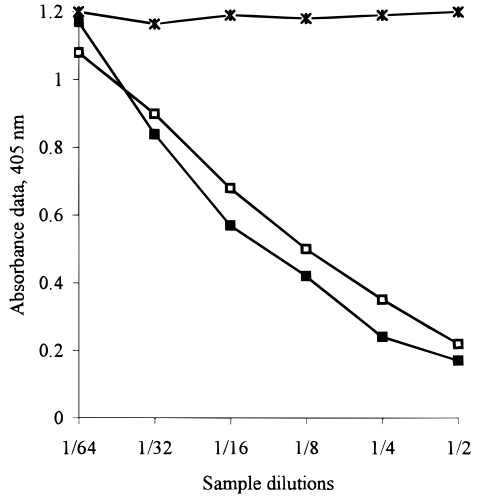

Figure 2. Antiserum Pp44 cross-reactivity with caseins: proteinase from *C. parasitica* (*), whole casein (□), and curd sample produced with *C. parasitica* (■) were diluted in TCS, pH 7.

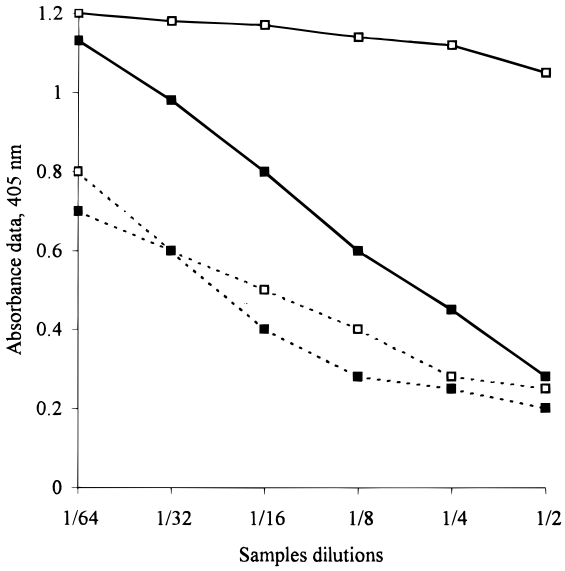

Figure 3. Influence of the pH parameter on the immune detection of porcine pepsin in curd sample from a soft cheese preparation. Curd samples were manufactured with either the proteinase from *C. parasitica* (□) or porcine pepsin (■). Curd samples were prepared and diluted in either TCS pH 6 (—) or pH 7 (- - -).

with caseins or with proteinase from *C. parasitica.* Only the residual porcine pepsin present in cheese sample inhibited the immune reaction at a level of ~75%. This percentage of inhibition corresponds to ~163 ng of porcine pepsin/g of cheese, which represents ~14% of the total amount of porcine pepsin added to milk. The total recovery of porcine pepsin detected at the end of cheese-making in both curd and whey represented ~94% of the total amount added to milk, so most of the porcine pepsin was detected using the optimized inhibition ELISA. Changes in pH parameter during both cheese sample preparation and ELISA test permit us to improve the detection of both free and adsorbed porcine pepsin on caseins. TCS solution seems to be more effective at pH 6 than at pH 7 for detecting residual porcine pepsin in cheeses without affecting total dissolution of caseins. Retention of rennet in the curd after manufacture has been demonstrated to depend on the conditions during manufacture, including

pH of gel at cutting, curd pH at whey drainage, cheese variety, and type of coagulant (Creamer et al., 1985; Singh and Creamer, 1990). For porcine pepsin, it was demonstrated that the diminution of curd pH from 6.6 to 6.0 induces an increase of its retention on caseins (Holmes et al., 1977; IDF, 1990). Thus, it is plausible that charges of caseins at pH 6 are involved in the retention of porcine pepsin, which reduces the interference of the caseins with the antiserum Pp44 at pH 7. This makes possible the immune detection of the adsorbed porcine pepsin through its accessible epitopes in cheese sample. In addition, the ELISA test developed herein does not require previous extraction of porcine pepsin from cheese samples.

In summary, the observed results for porcine pepsin detection proved the usefulness of the optimized inhibition ELISA for both whey and cheese samples from soft cheese preparation. This ELISA was also sufficiently specific and sensitive for the detection of low amounts of residual porcine pepsin in curd sample; it can be used for routine analyses. During this study, we demonstrated the importance of the pH parameter for detecting residual porcine pepsin in a soft cheese sample. Furthermore, the antiserum Pp44 can be used to detect both native and heat-denatured porcine pepsin. Further studies should be carried out to test the reliability of the present optimized ELISA on curd samples from pressed and pressed−cooked cheeses.

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