



Food and Agricultural Immunology

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/cfai20>

Detection of porcine pepsin in rennet mixtures used in cheese making by ELISA

A. S. Zahran ^a, S. A. Madkor ^a, M. M. Hewedi ^b, M. El Batawy ^b, A. M. A. Ibrahim ^b & C. J. Smith ^{c d}

^a Department of Dairy and Food Science, Faculty of Agriculture, University of El-Minia, Egypt

^b Department of Dairy Technology, Faculty of Agriculture, University of Cairo at Fayoum, Egypt

^c North East Wales Institute, Connah's Quay, Clwyd, CH5 4BR, UK

^d Cortecs Diagnostics Ltd, Techbase 1, Newtech Square, Deeside Industrial Park, Clwyd, CH5 2NT, UK

Published online: 16 Sep 2008.

To cite this article: A. S. Zahran, S. A. Madkor, M. M. Hewedi, M. El Batawy, A. M. A. Ibrahim & C. J. Smith (1994) Detection of porcine pepsin in rennet mixtures used in cheese making by ELISA, *Food and Agricultural Immunology*, 6:1, 85-93, DOI: [10.1080/09540109409354816](http://dx.doi.org/10.1080/09540109409354816)

To link to this article: <http://dx.doi.org/10.1080/09540109409354816>

PLEASE SCROLL DOWN FOR ARTICLE

Taylor & Francis makes every effort to ensure the accuracy of all the information (the "Content") contained in the publications on our platform. However, Taylor & Francis, our agents, and our licensors make no representations or warranties whatsoever as to the accuracy, completeness, or suitability for any purpose of the Content. Any opinions and views expressed in this publication are the opinions and views of the authors, and are not the views of or endorsed by Taylor & Francis. The accuracy of the Content should not be relied upon and should be independently verified with primary sources of information. Taylor and Francis shall not be liable for any losses, actions, claims, proceedings, demands, costs, expenses, damages, and other liabilities whatsoever or howsoever caused arising directly or indirectly in connection with, in relation to or arising out of the use of the Content.

This article may be used for research, teaching, and private study purposes. Any substantial or systematic reproduction, redistribution, reselling, loan, sub-licensing, systematic supply, or distribution in any form to anyone is expressly forbidden. Terms & Conditions of access and use can be found at <http://www.tandfonline.com/page/terms-and-conditions>

Detection of Porcine Pepsin in Rennet Mixtures Used in Cheese Making by ELISA

A. S. ZAHRAN,¹ S. A. MADKOR,¹ M. M. HEWEDI,² M. EL BATAWY,²
A. M. A. IBRAHIM² AND C. J. SMITH³

¹Department of Dairy and Food Science, Faculty of Agriculture, University of El-Minia, Egypt; ²Department of Dairy Technology, Faculty of Agriculture, University of Cairo at Fayoum, Egypt; ³North East Wales Institute, Connah's Quay, Clwyd CH5 4BR, UK

(Received for publication 12 September 1993)

An ELISA for the detection of porcine pepsin has been developed. Porcine pepsin was detected using this assay at concentrations of $\geq 1 \mu\text{g ml}^{-1}$. Other cheese renneting agents, such as bovine rennet or *Mucor miehei* rennet did not cross-react in the assay either separately or in mixtures. The ELISA was used for the determination of porcine pepsin at concentrations of 1% ($10 \mu\text{g ml}^{-1}$) and over in combination with bovine rennet/*M. miehei* rennet mixtures. The effects on the assay of samples which had been subjected to a pH range between 5.0 and 7.0 (which may be encountered during the storage of rennet mixtures and in cheese making procedures), were tested. Similarly pre-incubation of porcine pepsin in the presence of NaCl up to 10%, conditions encountered in certain salty cheese preparations, had no subsequent effect on the sensitivity of the assay when such samples were tested. Milk proteins showed no cross-reactivity with the anti-porcine pepsin and increasing concentrations of casein to 75 mg ml^{-1} caused no significant inhibition

Keywords: Porcine pepsin, rennet, ELISA, cheese

INTRODUCTION

Porcine pepsin is one of several renneting agents which can be used as coagulating enzymes in the dairy industry for cheese making. Trials using coagulating enzymes other than bovine rennet have shown that porcine pepsin can be used in the making of Cheddar cheese as well as the preparation of cheeses with a short ripening period (Minark *et al.*, 1978; Mulvihill *et al.*, 1979). However, for religious reasons, porcine pepsin can not be used as a renneting agent in many countries. Such countries are therefore reluctant to give legal clearance for the presence of porcine pepsin in commercial coagulating mixtures used

Correspondence to C. J. Smith. Present address: Cortecs Diagnostics Ltd, Techbase 1, Newtech Square, Deeside Industrial Park, Clwyd CH5 2NT, UK.

in cheese making and are reluctant to import cheeses which may have been produced using porcine pepsin as the renneting agent.

Few analytical methods have been developed for the detection of residual milk coagulating enzymes in cheese. The available methods generally involve the diffusion of the enzyme into a gel containing *k*-casein (Lawrence & Sanderson, 1969; Holmes *et al.*, 1977). These methods failed, however, to differentiate between rennin and rennin-like enzymes in coagulating mixtures used in cheese making. Other investigators (Harboe, 1985; Rauch *et al.*, 1989) have proposed methods for the assessment of residual milk clotting enzymes but these techniques were not sensitive enough to determine the origin of the rennet. Immunoassay techniques have yielded more reliable results. Matheson (1981) developed an immunodiffusion assay for the detection of chymosin in the presence of other milk coagulating enzymes. Elliott and Emmons (1971) managed to detect chymosin using a passive, indirect haemagglutination assay. Sandwich enzyme immunoassays have been found to be very specific and sensitive (Morris & Clifford, 1985; Vaag & Munck, 1987). Using this technique Rauch *et al.* (1989) managed to detect *Mucor Miehei* proteinase (from the commercial preparation Fromase) at very low concentrations (approx. 50 ng ml⁻¹).

ELISA is an analytical technique which has been found to be sensitive, rapid and reliable (Ayob *et al.*, 1988; Murphy *et al.*, 1992). El-Batawy *et al.* (1992) have developed an ELISA for the detection of porcine pepsin. The assay was sensitive enough to detect porcine pepsin in the range 0.001–1 mg of protein ml⁻¹. Such a method would be valuable in monitoring the presence of porcine pepsin that may have been added to commercial renneting preparations for economic or technological reasons.

The objective of this study was to demonstrate the ability of the ELISA to detect porcine pepsin in rennet mixtures and also to investigate the effects of the different conditions arising during cheese making on the reliability and sensitivity of this assay. These studies are intended to form a basis for further analyses on the presence of porcine pepsin in cheese samples.

MATERIALS AND METHODS

Reagents and Buffers

Porcine pepsin, bovine rennet and *M. miehei* rennet were purchased from Sigma Chemical Co. Ltd. (Poole, Dorset, UK). Polystyrene, flat-bottomed 96-well microtiter plates were purchased from Dynatech Laboratories Inc. (Immulon II, Chantilly, USA). Carbonate-bicarbonate buffer, 0.05 M, pH 9.6, was used as the coating buffer. The wash buffer used in the assay was phosphate-buffered saline (PBS) pH 7.8, containing 2.92% NaCl, 5% Tween 80 and 0.01% thimerosal. The substrate for the enzyme, horseradish peroxidase (HPR), contained 0.012% (v/v) H₂O₂ in citrate buffer (pH 4.0) with 6.0 mg of 2-2'-azino-bis (3-ethylbenzthiazoline sulphonic acid) (ABTS) ml⁻¹.

Methods

Antiserum production. Rabbits were immunized with a solution of porcine pepsin (2.0 mg ml⁻¹) mixed with an equal volume of Freund's complete adjuvant. The immunization schedule used was as described by Murphy *et al.* (1992).

Sensitization of ELISA plates. Polystyrene microtiter plates with flat-bottomed wells were sensitized by coating with porcine pepsin (100 µl well⁻¹, 5.0 µg ml⁻¹) in coating buffer using an overnight incubation at 4°C. At the end of the incubation period, plates were washed three times in carbonate-bicarbonate buffer, pH 9.6. The plates were then placed in

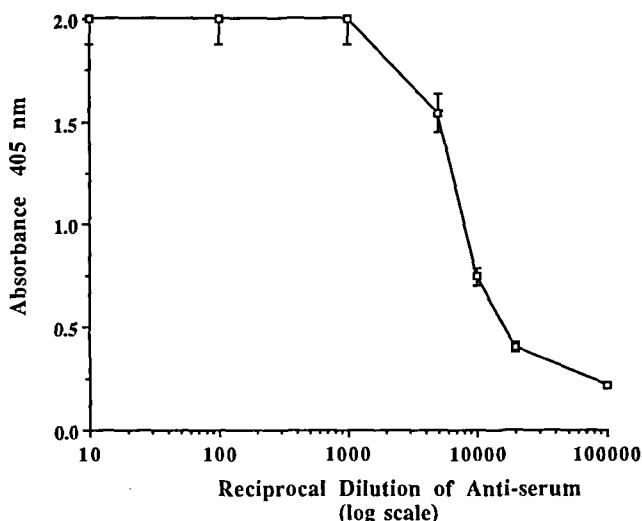


FIG. 1. Titration of serum samples suitable for use in the assay system.

a fan-ventilated incubator at 37°C for 2 h. Dried plates were sealed with tape and stored at 4°C until used.

ELISAs for the detection of antibodies were carried out according to a modification of the method of Voller *et al.* (1976). Briefly, the serum was diluted (1:5000) in 0.01 M-PBS, pH 7.8. Diluted samples (100 μ l) were added to previously prepared coated plates and incubated with constant shaking for 1 h at ambient temperature. The wells were then washed five times with wash buffer (PBS) and blot dried. Peroxidase-labelled anti-rabbit IgG (Sigma Chemical Co. Ltd, UK), diluted 1:200 in wash buffer was then added (100 μ l well⁻¹). The plates were incubated for 1 h with constant shaking, then washed five times with wash buffer and blot dried. The enzyme substrate (100 μ l well⁻¹) was then added. The plates were incubated with constant shaking for 10 min after which the optical density at 405 nm was measured using a Multiskan plate reader. Blank wells were treated in the same way as the test wells except that wash buffer replaced the serum samples.

Indirect competitive ELISA. The method of Murphy *et al.* (1992) was used. Antigen solutions (50 μ l), ranging in concentration from 0.001 to 1.0 mg ml⁻¹, were dispensed into the wells of the ELISA plate. Antibody (50 μ l) was added to each of the wells containing antigen solution or dilution buffer (control wells), and the plates incubated for 1 h at ambient temperature.

The subsequent steps were the same as for the antibody detection ELISA described above. The absorbance, at 405 nm, of each well was read against a blank of ABTS solution. The absorbance of negative controls was subtracted from all readings. The reduction in absorbance as a result of the presence of antigen was calculated and expressed as a percentage inhibition:

$$\% \text{Inhibition} = 100 \left[\frac{(A_{405 \text{ nm}}^{\text{antibody} + \text{PBS}}) - (A_{405 \text{ nm}}^{\text{antibody} + \text{antigen}})}{(A_{405 \text{ nm}}^{\text{antibody} + \text{PBS}})} \right] \times 100$$

The percentage inhibition was calculated for each concentration of each enzyme solution used.

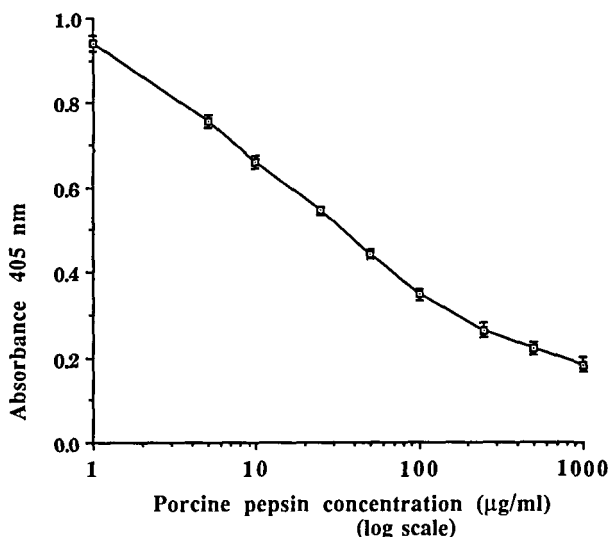


FIG. 2. Standard curve of a competitive ELISA for porcine pepsin determination. The points are the means of 10 replicate results. The standard deviation for each of the points was $< 5.2\%$.

RESULTS AND DISCUSSION

Antisera produced in rabbits by the method described was pooled and titrated. The pooled samples were shown to be suitable for use in the assay system at a dilution of 1:5000 in 0.01 M-PBS, pH 7.8 (Figure 1). Using the pooled antiserum, it was possible to measure different concentrations of porcine pepsin by carrying out a competitive ELISA (Figure 2). The assay was able to detect porcine pepsin at concentrations ranging from 1 to 1000 $\mu\text{g ml}^{-1}$. One of the applications of immunoassays in the dairy industry is the detection of porcine pepsin in rennet mixtures. Using the technique described, it was possible to measure the levels of porcine pepsin in specially prepared mixtures of porcine pepsin, bovine and *M. miehei* rennet (Figure 3). The precision of the standard curve was estimated by calculating the percentage coefficient of variance (%CV) of inter-assay variation over 10 replicates. The %CV for all the standard samples in the assays was $< 5.2\%$ (Figure 4). The precision and reproducibility of the competitive inhibition assay in measuring the porcine

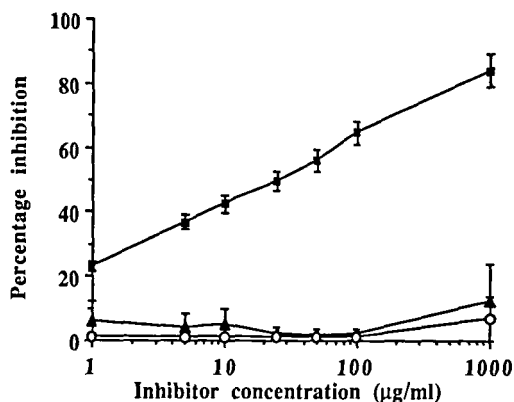


FIG. 3. Inhibition and cross-reactivity assays using anti-porcine pepsin antiserum. (■) Porcine pepsin; (▲) bovine rennet; (○) and *M. miehei* rennet.

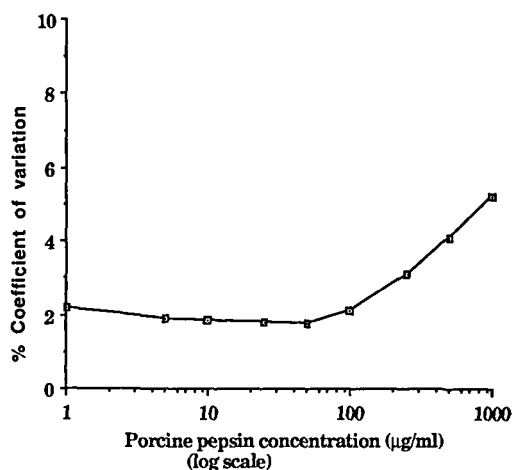


FIG. 4. Precision profile for standard assay.

pepsin content of mixtures of the three rennets was slightly higher (Table 1). A comparison of the absorbance of known concentrations of porcine pepsin in rennet mixtures with the absorbance of standard porcine pepsin alone revealed no significant differences (Table 1).

The effect of different parameters on the stability of the analyte and the subsequent assay were investigated. In cheese making the procedure may include changes in both pH and salinity to which the antigen, porcine pepsin, might be exposed. Thus, the immunoreactivity of samples of porcine pepsin following treatment at different pHs or different salt concentrations was investigated. The sensitivity of the assay was not affected by changing the pH within the range of pH used in cheese-making procedures (pH 5.0–7.0) (Figure 5). A slight decrease in sensitivity at pH 4.5 was noted. The optimum pH for rennet activity, the main milk coagulating enzyme is between 5.5 and 5.8 (McMahon & Brown, 1984), hence the assay should provide a satisfactory means of measuring porcine pepsin from samples which have been exposed to these pH values.

Sodium chloride is normally added to milk before renneting when making the traditional Egyptian Domiati cheese. NaCl is normally added at concentrations of up to 10% (El-Koussy *et al.*, 1984; Magdoub *et al.*, 1984). Different concentrations of NaCl (2–10%) were added to porcine pepsin solutions (1 mg ml⁻¹ in phosphate buffer, pH 7.0). The saline-enzyme solutions were incubated in a water bath at 37°C for 3 h. Enzyme solutions then were dialyzed for approximately 18 h at 4°C to remove the NaCl before assaying the dialyzed solution for porcine pepsin. Results (Figure 6) show that pre-incubation of porcine pepsin in the presence of different concentrations of NaCl did not affect the inhibition assay.

TABLE 1. Assay precision and reproducibility of a competitive ELISA for porcine pepsin detection in bovine rennet/*M. miehei* rennet mixture determined from the standard curve

Porcine pepsin in rennet mixture (%) ^a	Mean optical density observed (405 nm)	Standard deviation	Expected optical density (405 nm)	%CV
2.5	0.541	0.029	0.545	5.36
10	0.332	0.016	0.348	4.82
50	0.237	0.017	0.220	7.17

^aThe concentration of bovine rennet and *M. miehei* was 1.0 mg ml⁻¹ in each assay. All assays were run in quadruplicate and the results given are the mean from 10 assays.

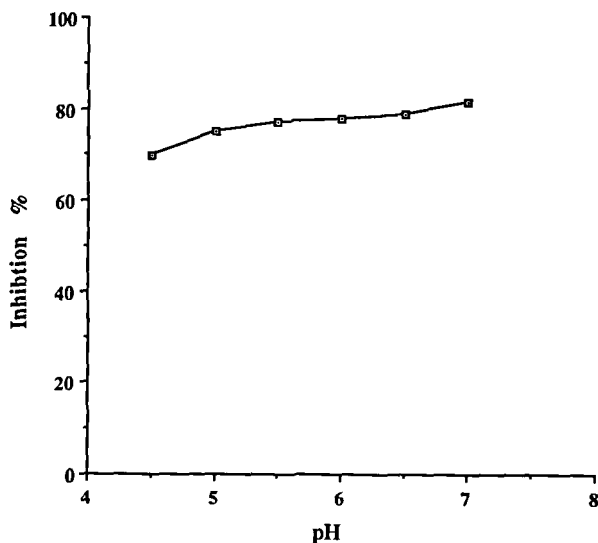


FIG. 5. Effect of incubation of porcine pepsin for 3 h at different pH values on the inhibition assays using anti-porcine pepsin and antiserum.

Tests on the effect of milk proteins on the assay were carried out using casein and whey proteins, either separately or as mixtures (Table 2). Casein (2.8%, w/v) and whey protein (0.8%, w/v) were added to porcine pepsin (1 mg ml^{-1} in phosphate buffer, pH 7.0). These mixtures were incubated at 37°C for 3 h, then the ELISA was carried out. The cross-reactivity of casein and whey proteins was insignificant, so milk proteins at concentrations normally found in milk had no effect on the sensitivity of the assay (Table 2). Ultrafiltration is now used in the dairy industry as a preparative technique prior to cheese making to concentrate the milk proteins (El-Shibiny *et al.*, 1984; Green *et al.*, 1985), therefore, the effect of more concentrated casein solutions was also studied. Increasing the concentration of casein as much as around 4 times above the normal concentration found in milk had no effect on the pepsin ELISA (Figure 7). Similarly, when concentrated whey proteins up to 10.0 times the concentration found in milk were tested no effect was observed (Figure 7).

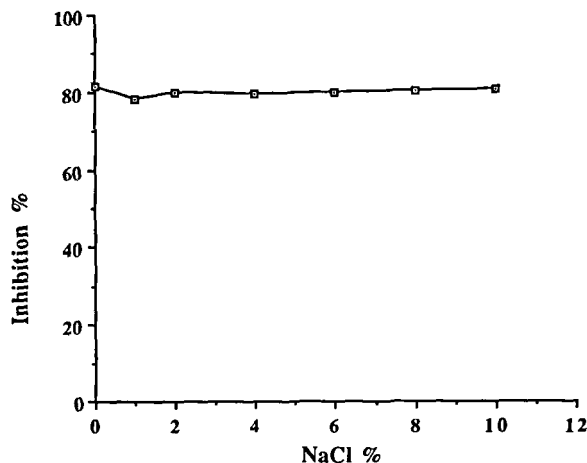


FIG. 6. Effect of pre-incubation of porcine pepsin for 3 h in the presence of different concentration of NaCl on the inhibition assays using anti-porcine pepsin antiserum.

TABLE 2. Effect of porcine pepsin/milk protein mixtures on the inhibition assay

Protein mixture	Inhibition (%)
Porcine pepsin ^a	82.54
Porcine pepsin/casein ^b	81.61
Porcine pepsin/whey ^c	83.02
Porcine pepsin/whey plus casein	84.82

^aPorcine pepsin concentration: 1000 $\mu\text{g ml}^{-1}$.^bCasein 2.8% (w/v) 28 $\mu\text{g ml}^{-1}$.^cWhey protein 0.8% (w/v) 8 $\mu\text{g ml}^{-1}$.

For technical and economical reasons, porcine pepsin may be used in the manufacture of different types of cheese. Minark *et al.* (1978) and Mulvihill *et al.* (1979) reported that porcine pepsin could be used in the manufacture of Cheddar cheese and cheeses with a short ripening period. Wahba & El-Abbassy (1981) manufactured Domiati cheese using different milk clotting enzymes. They found that cheeses made with calf rennet or calf rennetin 1:1 mixture with sheep or porcine pepsin gave the best organoleptic scores. As far as the dairy industry in some countries is concerned, detection of porcine pepsin in imported rennet mixtures is very important. Therefore, cross-reactivity assays were carried out using different combinations of bovine rennet and porcine pepsin (1:1), *M. miehei* rennet and porcine pepsin (1:1) and a mixture of the three enzymes (1:1:1). No significant inhibition was observed (Figure 8) with either bovine rennet or *M. miehei* rennet over the concentration range tested. However, in the presence of porcine pepsin the assay gave results for mixtures of enzymes equivalent to those obtained with porcine pepsin alone. This showed that the ELISA could be used to detect porcine pepsin in rennet mixtures.

In conclusion, the specificity of the assay for porcine pepsin content is clearly demonstrated. Studies with different combinations of rennets demonstrated that the assay is unaffected by the presence of bovine and *M. miehei* rennet alone or in mixtures with porcine pepsin. Preliminary results showed that the level of other proteins which may be present in cheese samples did not affect the assay, nor did prior exposure of coagulating mixtures to sodium chloride or different pH values within the levels expected in cheese

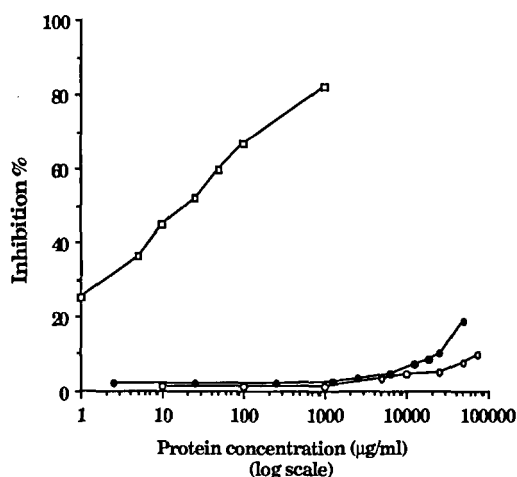


FIG. 7. Results of inhibition and cross-reactivity assays using anti-porcine pepsin antiserum. (□) Porcine pepsin; (●) casein; and (○) whey.

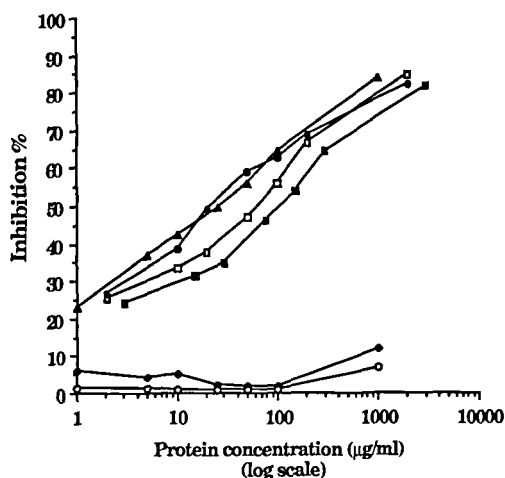


FIG. 8. Results of inhibition and cross-reactivity assays using anti-porcine pepsin antiserum. (□) Porcine pepsin; (◆) bovine rennet; (○) *M. miehei* rennet; (▲) porcine pepsin/bovine rennet 1:1; (●) porcine pepsin/*M. miehei* rennet 1:1; (■) mixtures of porcine pepsin/bovine rennet/*M. miehei* rennet 1:1:1. Each point is the mean of results from five separate assays carried out in triplicate.

samples. Further studies are currently in progress to determine the usefulness of the assay for the detection of porcine pepsin in the cheese matrix.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the financial support of the British Council which permitted A. S. Z., M. M. H. and S. A. M. to work at the North East Wales Institute (NEWI). The authors also wish to acknowledge the financial support of the Egyptian Students' Bureau which permitted A. I. to work at NEWI during her PhD studies.

REFERENCES

- AYOB, M.K., RITTENBURG, J., ALLEN, J.C. & SMITH, C. J. (1988) Development of a rapid enzyme-linked immunosorbent assay (ELISA) for gliadin determination in food, *Food hydrocolloids*, **2**, 39–49.
- EL-BATAWY, M., ZAHKAN, A. S., MADKOR, S. A. & SMITH, C. J. (1993) Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of porcine pepsin as milk coagulating enzyme, *Food and Agriculture Immunology*, **5**, 57–60.
- EL-KOUSSY, L., EWAIS, S. & HEFNAWY, S. (1984) Manufacture of half cream soft cheese. III. Effect of salting method, *Egyptian Journal of Dairy Science*, **12**, 259–266.
- ELLIOTT, J. A. & EMMONS, D. B. (1971) Rennin detection in cheese with the passive indirect hemagglutination test, *Canadian Institute of Food Technology Journal*, **4**, 16–18.
- EL-SHIBINY, S., ABD-EL-SALAM, M. H., MAHFOUZ, M. B. & EL-ALAMY, H. A. (1984) Effect of coagulation method on composition and quality of skim milk cheese (Karish) made by ultrafiltration technique, *Egyptian Journal of Dairy Science*, **12**, 243–250.
- GREEN, M. L. (1985) Effect of milk pretreatment and making conditions on the properties of Cheddar cheese from milk concentrated by ultrafiltration, *Journal of Dairy Research*, **52**, 555–564.
- HARBOE, M. K. (1985) Commercial aspects of aspartic protease, in *Aspartic Proteinases and Their Inhibitors* (KOSTKA, V., Ed.) W. De Gruyter, Berlin, pp. 537–549.
- HOLMES, D. G., DUERSCH, J. W. & ERNSTROM, C. A. (1977) Distribution of milk clotting enzymes between curd and whey and their survival during Cheddar cheese making, *Journal of Dairy Science*, **60**, 862–869.
- LAWRENCE, R. C. & SANDERSON, W. B. (1969) A micro-method for the quantitative estimation of rennets and other proteolytic enzymes, *Journal of Dairy Research*, **36**, 21–29.
- MAGDOUB, M. N. I., SHEHATA, A. E., FAYED, E. O. & HOFI, A. A. (1984) Effect of added salt and capsium tincture on lactic acid bacteria in pickled Domiati cheese, *Egyptian Journal of Dairy Science*, **12**, 209–218.

- MATHESON, A. R. (1981) The immunochemical determination of chymoshin ctivity in cheese, *New Zealand Journal of Dairy Science and Technology*, **16**, 33–41.
- MCMAHON, D. & BROWN, R. J. (1984) Enzymic coagulation of casein micelles: a review, *Journal of Dairy Science*, **67**, 919–929.
- MINARK, R., TEPLY, M. & DVORAK, F. (1978) Possibilities of the application of various enzymes with renneting capacity in the production of cheese, in *International Dairy Congress*, Vol. E, pp. 437–438.
- MORRIS, B. A. & CLIFFORD, M. N. (1985) *Immunoassays in Food Analysis*, Elsevier, London.
- MULVIHILL, D. M., COLLIER, T. M. & FOX, P. F. (1979) Manufacture of coagulant-free cheese with piglet gastric proteinase, *Journal of Dairy Science*, **62**, 1567–1569.
- MURPHY, J. F., DAVIES, D. H. & SMITH, C. J. (1992) The development of enzyme-linked immunosorbent assays (ELISA) for the catecholamines adrenalin and nonadrenalin, *Journal of Immunological Methods*, **154**, 88–89.
- RAUCH, P., HOHELL, I., BERANKOVA, E. & KAS, J. (1989) Sandwich enzyme immunoassay of *Mucor miehei* proteinase (Fromase) in cheese, *Journal of Dairy Research*, **56**, 793–797.
- VAAG, P. & MUNCK, L. (1987) Immunochemical methods in cereal research and technology, *Cereal Chemistry*, **64**, 59–72.
- VOLLER, A., BIDWELL, D. & BARTELL, A. (1976) Microplate enzyme immunoassays for the immunodiagnosis of virus infections, in: *N. Rose and H. Friedman (Eds) Manual of Clinical Immunology*, (ROSE, N. & FRIEDMAN, H. Eds), American Society for Microbiology, Washington, DC, USA, p. 506.
- WAHBA, A. & EL-ABBASSAY, F. (1981) Manufacture of Domiati cheese with rabbit, sheep and porcine pepsins and calf rennet, *Egyptian Journal of Dairy Science*, **9**, 11–17.