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Characterization of the proteolytic activity of starter cultures of *Penicillium roqueforti* for production of blue veined cheeses

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Abstract

Thirty strains of *Penicillium roqueforti* used for the production of blue cheeses were examined for proteolytic activity by agar diffusion on casein agar, by the azocasein test and by capillary zone electrophoresis (CE). Distinct differences were seen between the strains by all three methods applied and the 30 strains could be subdivided in three groups being significantly different in their proteolytic activity as measured by the agar diffusion test. The quantitative differences seen in the agar diffusion test were confirmed by the azocasein test. However, a negative result on casein agar, i.e., no clearing of the agar was observed for one strain while it showed low proteolytic activity in the azocasein test. CE proved to be a valuable method for revealing qualitative differences between strains of *P. roqueforti* in the breakdown of casein. Three strongly proteolytic strains broke down the specific casein fractions differently: one strain broke down βA1-casein faster than βA2-casein, the second preferred α_{s1}-casein while the last strain broke down the casein fractions at equal rates. For a strain with medium proteolytic activity, the degradation of casein was seen by the appearance of a peak with migration time similar to α_{s1}-I casein, a peptide normally produced by chymosin.

Keywords: *Penicillium roqueforti*; Blue cheese; Proteolytic activity; Capillary electrophoresis (CE)

1. Introduction

The selection of *Penicillium roqueforti* strains for the manufacture of Danablu and other blue-veined cheeses is often based on experience rather than on detailed studies of their technological characteristics, e.g. enzymatic activity. The proteolytic activity of the strain, and thereby the degree of proteolysis, is important for the development of a cheese with an optimal texture, water binding capacity and flavour (Kinsella and Hwang, 1976).

In blue-veined cheeses, the breakdown of casein is very intense compared to other ripened cheeses (Marcos et al., 1979; Trieu-Cuot and Gripon, 1983; Hewedi and Fox, 1984; Gonzalez de Llano et al.,

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1995). At the end of ripening, α_{s1}-casein has almost disappeared (Marcos et al., 1979; Trieu-Cuot and Gripon, 1983) and also β-casein is broken down to a large extent (Marcos et al., 1979; Trieu-Cuot and Gripon, 1981; Trieu-Cuot et al., 1982; Trieu-Cuot and Gripon, 1983). This extensive breakdown of the caseins is primarily due to the action of proteinases from *P. roqueforti* (Desmazeaud et al., 1976; Gripon et al., 1977; Coghill, 1979; le Bars and Gripon, 1981; Bracq et al., 1997).

The proteolytic activity of *P. roqueforti* has been determined by various methods like agar diffusion tests in casein agar (Modler et al., 1974; López-Díaz et al., 1996), breakdown of azocasein measured by spectrophotometry (Engel and Teuber, 1988), casein degradation measured by the Folin colorimetric method (Niki et al., 1966; Stepaniak et al., 1980) and determination of water, TCA and phosphotungstic acid soluble nitrogen in cheeses and in cheese model curds (Fernandez-Salguero et al., 1989; Farahat et al., 1990; Zarmpoutis et al., 1996). Comparative studies of the various methods seem not to have been reported.

A new method, capillary zone electrophoresis (CE), has been described for qualitative studies of casein breakdown by different enzymes. It has been used successfully in the determination of the action of chymosin and plasmin on casein (Kristiansen et al., 1994; Recio et al., 1997). The degradation of the various casein fractions and the subsequent production of peptides during ripening of Feta, Mozzarella and Danbo cheeses has also been investigated by CE (Otte et al., 1997). In CE, separation is based on differences in the charge-to-mass ratio and it has the advantages of using very small sample sizes, being easy to perform and combining separation and quantification in one step (Kristiansen et al., 1994; Otte et al., 1997). The use of CE to characterize the proteolytic activity of starter cultures, even though it gives detailed information on the breakdown of the individual casein fractions, has not been described previously.

The three methods applied in this study have different sensitivities and thereby give different information. A positive result in the agar diffusion test, i.e. a clearing of the casein agar, requires a complete degradation of the casein (unpublished results). The azocasein test is more sensitive (Engel and Teuber, 1988), but it gives only quantitative information, while CE gives both quantitative and qualitative data.

By using the three methods mentioned, it was the objective of the present study to quantify the proteolytic activity of a large number of commercial strains of *P. roqueforti* by the agar diffusion test and by the azocasein test and to use CE to investigate qualitative differences in the breakdown of casein by selected strains of *P. roqueforti*.

2. Materials and methods

2.1. Fungi

The 30 strains of *Penicillium roqueforti* included are shown in Table 1. They were maintained as stock cultures of dried conidia. The conidia were produced on DG-sucrose, in g/l: bactopeptone 5.0 (Difco, Detroit, MI, USA), D(+)-glucose-monohydrate 10.0 (Merck, Darmstadt, Germany), agar 20.0, $Mg_2SO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 1.0, sucrose 360 and trace element solution 1 ml. The trace element solution contained $ZnSO_4 \cdot 7H_2O$ 1 g/100 ml and $CuSO_4 \cdot 5H_2O$ 0.5 g/100 ml. The conidia were harvested from the agar surface after 3 weeks incubation at 25°C and dried in a desiccator for 24 h at ambient temperature, under aseptic conditions, and kept under refrigeration.

2.2. Agar diffusion test

The agar diffusion test in casein agar was carried out according to Lawrence and Sanderson (1969) with slight modifications. Cheese agar prepared as described below was inoculated with 1 ml of a conidial suspension in salt peptone solution (SPO), g/l: peptone 1.0 (Difco), NaCl 8.5, $Na_2HPO_4 \cdot 2H_2O$ 0.3, Tween 80 10 ml (Merck) (10^7 conidia per ml) and incubated at 25°C for 5 days. The concentration of conidia in SPO was estimated by microscopy. At the end of incubation, agar plugs with a diameter of 10 mm were transferred to a test tube with casein agar, prepared as described below, and placed with the mould growth upwards. After 3 days of incubation at 25°C, the depth of the clearing zone was measured. All tests were repeated on two separate occasions in triplicate and the mean value and standard deviation reported.

Table 1
Proteolytic activity of 30 commercial strains of *P. roqueforti* for blue cheese production measured by agar diffusion test on casein agar after five days of incubation at 25°C

Strain of *P. roqueforti*	Origin of the strain	Clearing zone[a] (mm)
roq 1	AJL[b]	0
roq 11	AJL	0
roq 14	AJL	0
roq 34	Visby[c]	0
PJ	Visby	0
roq 4	AJL	6.3±0.5
roq 5	AJL	6.7±0.5
roq 31	Visby	7.3±0.5
roq 32	Visby	8.0±0.0
PA	Visby	8.0±0.0
fr 7	French blue cheese[f]	8.0±0.0
roq 33	Visby	9.0±0.0
roq 19	AJL	9.0±0.0
roq 8	AJL	9.3±0.5
fr 6	French blue cheese	9.3±0.9
Mauri	Mauri[d]	9.3±0.9
fr 2	French blue cheese	10.0±0
roq 35	Visby	10.0±0
roq 36	Visby	10.0±0
roq 37	Visby	10.0±0.8
roq 2	AJL	10.0±0.8
PV	Visby	10.3±0.5
CSL	CSL[e]	10.7±0.5
fr 1	French blue cheese	11.2±0.2
roq 18	AJL	11.2±0.2
fr 3	French blue cheese	11.5±0.4
roq 15	AJL	11.7±0.2
fr 4	French blue cheese	11.7±0.5
fr 5	French blue cheese	11.7±0.5
roq 38	Visby	13.7±0.5

[a] The results are expressed as millimetre depth of the clearing zone. The strains are listed in order of increasing proteolytic activity. The mean value and standard deviation are reported for triple determinations carried out on two different occasions.
[b] Alfred Jørgensen Laboratory A/S, Copenhagen, Denmark.
[c] Laboratorium Visby, Tønder, Denmark.
[d] Mauri Laboratories, Dorset, England.
[e] Centro Sperimentale Del Latte, Milan, Italy.
[f] Isolates from french blue cheeses. The remaining strains are commercial cultures for production of Danablu.

The cheese agar was prepared by autoclaving 1.5 g bacto agar (Difco) in 500 ml demineralized water for 20 min at 121°C and then mixing it with 400 g unsalted Danablu cheese received from a Danablu producing dairy and cooked for 30 min before use to inactivate the primary starter culture and the *P. roqueforti* present. The casein agar contained in g/l: casein acc to Hammarsten 10.0 (Merck), calcium hydroxide 0.3 (Merck), calcium chloride 0.20 and agar 15.0 (Merck). The pH was adjusted to 5.8. The substrate was distributed in 10-ml portions into test tubes and autoclaved for 20 min at 121°C.

2.3. Azocasein test

The azocasein method used was a modified version of the method described by Engel and Teuber (1988). Czapek-Dox broth (Difco), 200 ml, with 0.5% bactopeptone (Difco) and 0.1% trace element solution, pH 4.0, was inoculated with 2 ml of a conidial suspension in SPO (10^7 conidia per ml) and incubated at 25°C in a shaking water bath at 200 rpm (SBD50 Bio, Heto, Allerød, Denmark). After three days of incubation, the mycelia were removed by filtration through several layers of lens tissue (Joseph

paper, Struers KEBO Lab. A/S, Albertslund, Denmark). Then the mycelium-free broth was centrifuged at 10 000 × *g* (Sorvall RC-5B, Buch and Holm A/S, Herlev, Denmark) at 4°C for 20 min and sterile filtered (0.45 μm, Sartorius AG, Göttingen, Germany). This culture-free extract (CFE) were then concentrated approximately six times on a pressurized stirred ultrafiltration cell, Amicon 8400 (Amicon Corporation, Beverly, MA, USA), against a membrane with a cut-off value of 10 000 Daltons (PTGC 076 10, Millipore, Bedford, MA, USA). The concentrated CFE (0.1 ml), was mixed with 0.5 ml of a 1% azocasein (Sigma A-2765, St. Louis, MO, USA) solution in 0.1 M phosphate buffer, pH 5.0, 5.5 or 6.0, and incubated at 35°C for 1 h, followed by addition of 0.6 ml of a 10% TCA solution to stop the reaction. Then the tubes were centrifuged for 10 min at 7000 × *g*. The absorbance of the supernatant was read at 336 nm on a Shimadzu spectrophotometer (SpectraChrom, Broendby, Denmark) against a reaction mixture with 0.1 ml buffer instead of CFE. One unit of proteolytic activity (PAu) was defined as a 0.01 increase in absorbance during 1 h of incubation under the present assay conditions. The activity was expressed as PAu/ml CFE concentrate. The examinations were carried out on two separate occasions and the assays were made in triplicates. The mean values and standard deviations are reported.

2.4. Capillary electrophoresis

Czapek-Dox broth (100 ml, Difco), prepared with 0.1 M phosphate buffer, containing 1% irradiated (10 kGy) casein acc to Hammarsten (Merck), 0.1% trace element solution, pH adjusted to 5.5 or 6.0, was inoculated with 1 ml of a conidial suspension in SPO (10^6 conidia per ml). Incubation was at 25°C in a shaking water bath at 200 rpm (SBD50 Bio). Samples were taken after 3, 4 and 5 days of incubation. After centrifugation at 11 000 × *g* (Sigma 113, Struers KEBO Lab A/S) 25 μl of supernatant were mixed with 25 μl of sample buffer consisting of 8 M urea (Merck), 0.01 M dithioerythritol (Sigma D-8255), pH adjusted to 8.0, and a tripeptide marker (lys-tyr-lys, Sigma L-3271). The samples were left at ambient temperature for 1 h and analysed on a Waters Quanta 4000 capillary electrophoresis system (Waters Corporation, Milford, MA, USA), equipped with a fused-silica capillary column (J&W Scientific, Folsom, CA, USA, no. 160-2650) 50 μm I.D., 60 cm total length, 52.5 cm to the detector. The samples were injected hydrostatically for 25 s and separated at constant voltage. On-line detection was at 214 nm. The run buffer consisted of 0.01 M sodium phosphate buffer, pH 2.5, and added urea to 6 M and HMPC (hydroxy-propyl-methyl-cellulose, Aldrich-Chimie, Steinheim, Germany) to 0.02%. The examinations were carried out in duplicate on two separate occasions. The results were qualitatively similar hence only one set of electropherograms are shown.

3. Results and discussion

Initially all 30 strains of *P. roqueforti* were investigated for their proteolytic activity by agar diffusion in casein agar. The results obtained from the agar diffusion test are shown in Table 1. Fourteen strains showed a clearing zone between 10 and 14 mm and 11 showed a clearing zone between 6 and 10 mm. Four strains did not show any signs of clearing of the casein agar. In the investigation of López-Díaz et al. (1996) two commercial *P. roqueforti* strains and nine from a naturally ripened blue cheese were found to be nonproteolytic on skim milk agar. The reason could be that agar diffusion tests do not always provide the sensitivity required as the clearing can be very difficult to see especially in a medium such as skim milk. Therefore other methods likely to give a more correct picture of the proteolytic activity should be considered.

From the results obtained in the initial investigation, the following five strains of *P. roqueforti* characterized by low (roq 1), medium (roq 5) and high proteolytic activity (roq 2, PV and CSL) and at the same time being widely used in the dairy industry were investigated further by the azocasein method and by CE. Table 2 shows the proteolytic activity measured against azocasein at pH 5.0, 5.5 and 6.0. Except for roq 1, the highest proteolytic activity was observed at pH 5.0 followed by pH 5.5 and pH 6.0 where only approximately 40% of the activity was retained. Engel and Teuber (1988) investigated the proteolytic activity of 42 strains of *P. roqueforti* by the azocasein method and also found that the highest proteolytic activity was seen at the lowest pH examined, pH 5.3. Comparing Tables 1

Table 2
The proteolytic activity of five strains of *P. roqueforti* measured against azocasein at pH 5.0, 5.5 and 6.0 after three days incubation at 25°C

Strain of *P. roqueforti*[a]	pH 5.0 PAu/ml[b] CFE conc.	pH 5.5 PAu/ml CFE conc.	pH 6.0 PAu/ml CFE conc.
roq 1	174.6±83.6	192.1±53.1	70.1±8.1
roq 5	477.8±37.0	421.4±23.1	180.3±35.1
roq 2	1394.7±79.0	1130.3±61.8	609.3±160.9
PV	1028.9±98.6	814.7±80.0	409.4±90.6
CSL	828.9±39.0	644.3±95.7	293.3±151.3

[a] For definition of strains see Table 1.
[b] One unit of proteolytic activity (PAu) was defined as the increase in absorbence of 0.01 during 1 h of incubation under the present assay conditions and expressed as PAu/ml CFE (culture-free extract) concentrate. The mean value and standard deviations are reported for determination in triplicate carried out at two different occasions.

and 2, good agreement is seen between the results obtained by the two different methods although the five strains were not ranked in exactly the same order. Differences were observed in the ranking of the three highly proteolytic strains roq 2, PV and CSL, which could be due to the lower sensitivity and less detailed information obtained from the agar diffusion test. This is also seen for roq 1 which was considered to be nonproteolytic by the agar diffusion test, but showed a low activity in the azocasein test. By prolonging the incubation of roq 1 on the casein agar, a clearing was seen (results not included).

The differences as analysed by CE in the breakdown of casein at pH 5.5 after three days of cultivation for the same five cultures of *P. roqueforti* are illustrated in Fig. 1. The peaks are identified in Fig. 1a. The small differences seen in migration time are due to the use of buffers prepared at different dates, but the identification of the peaks were confirmed by calculating the migration time of the peaks relative to the migration time of the marker (results not shown).

The casein in the roq 1 and the roq 5 samples (Fig. 1a, b) was intact showing clearly the different casein components. This picture did not change during the five days incubation of roq 1 (results not shown). The most proteolytic strains, roq 2, PV and CSL, after three days of incubation had broken down the casein to a much larger extent than roq 5 and roq 1 (Fig. 1c, d, e). These three strains were able to degrade all the casein components present, but qualitative differences between the strains in the breakdown of the specific casein fractions were observed. Comparing the three strains, it is seen that the PV sample (Fig. 1d) apparently degrades the βA1-casein to a larger extent than the βA2-casein, which is not the case for roq 2 and CSL, indicating a higher activity of PV towards this fraction of the β-casein. The CSL strain (Fig. 1e) seems to degrade α_{s1}-casein a little faster than the β-casein fractions, while the roq 2 strain breaks down the casein fractions at equal rates (Fig. 1c). After 4 days of incubation, the casein was totally degraded by all three strains (results not shown).

For the roq 5 sample, casein degradation during the five days of incubation was demonstrated by the appearance of a peak with a migration time very similar to the peptide α_{s1}-I casein (Fig. 2), a peptide normally produced by the degradation of α_{s1}-casein by chymosin (Otte et al., 1997). The peptide was only seen for roq 5 probably due to its medium degradation rate.

At pH 6.0, the chromatogram for roq 1 and roq 5 showed the same profile as at pH 5.5 but for strains PV and CSL at pH 6.0, a recognizable casein profile was seen after three days incubation while strain roq 2 broke down the casein to a larger extent (results not shown). After 4 days incubation, the casein had almost disappeared in samples containing strains roq 2, PV and CSL (results not shown). The differences observed in the breakdown pattern between these strains at pH 5.5 were still seen at pH 6.0, but not as clearly. Comparing samples containing roq 2, PV and CSL at pH 5.5 and 6.0 after three days incubation, the casein in all the samples at pH 5.5 was broken down to a larger extent than at pH 6.0, indicating a higher activity or an increased production of proteinases at pH 5.5. This higher activity at the lower pH agrees with the results obtained by the azocasein method (Table 2) and also to the findings of Engel and Teuber (1988).

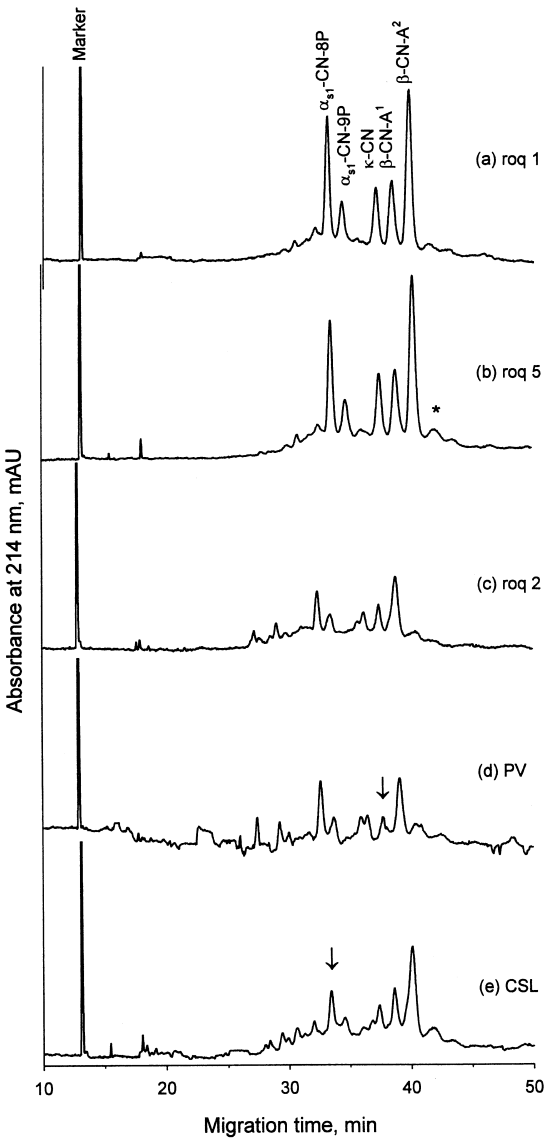

Fig. 1. CE profiles, absorbence at 214 nm versus time, of casein medium, pH 5.5, inoculated with the following strains of *P. roqueforti*: roq 1 (a), roq 5 (b), roq 2 (c), PV (d) and CSL (e) after incubation for 3 days at 25°C. (For definition of strains see Table 1). * Migration time similar to the peptide α_{s1}-I casein.

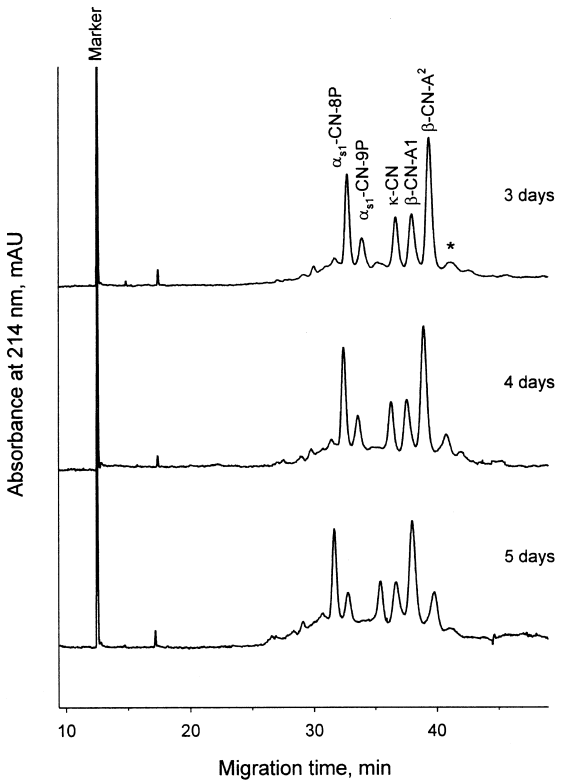

Fig. 2. CE profiles, absorbence at 214 nm versus time, of casein medium, pH 5.5, inoculated with the *P. roqueforti* strain roq 5 after incubation for 3, 4 and 5 days at 25°C. (For definition of strains see Table 1). * Migration time similar to the peptide α_{s1}-I casein.

The differences in proteolytic activity observed between the strains tested will have a major influence on the characteristics of the Danablu produced. It is known that strains with a low activity will give less mature cheeses with a milder taste and a longer shelf life, while the strains with a strong proteolytic activity will give a strong tasting, fast ripening cheese. The differences observed in the breakdown pattern of the caseins could give rise to different textures and water binding capacities in the cheeses and also to the production of different aroma compounds and peptides (e.g. bitter peptides.)

The three methods examined in this investigation all give useful results but they are of value in different circumstances. The method most suitable for a dairy would be the agar diffusion test. It is simple to prepare and to read and although important qualitative characteristics are not detectable, it is acceptable for the routine control of the proteolytic activity of new batches of *P. roqueforti*. However, care should be taken with very low proteolytic strains as could be seen with roq 1 in this investigation where the agar diffusion test was not sensitive enough to detect the proteolytic activity within the incubation time used. This could be

solved by prolonging the incubation on the casein agar. The azocasein test and CE give a more detailed picture of the proteolytic activity of the starter cultures, but are less suited for quality control purposes in dairies. According to the present work, CE seems to be a promising technique for characterization of the wide differences in the proteolytic activity of starter cultures of *P. roqueforti* important for the selection of cultures for the production of Danablu.

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