A C T A A L I M E N T A R I A P O L O N I C A
Vol. VI (XXX), No. 3 1980

LESZEK STEPANIAK
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LIPOLYTIC AND PROTEOLYTIC ACTIVITY OF *PENICILLIUM ROQUEFORTI, PENICILLIUM CANDIDUM* AND *PENICILLIUM CAMEMBERTI* STRAINS.

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Key words: *P. roqueforti, P. camembertt,* lipolytic enzymes, proteolytic enzymes

Studies carried out on the lipolytic and proteolytic activities of extra- and intracellular enzymes of chosen moulds' species showed higher lipolytic activity of P.roqueforti and P.candidum as compared to P.camemberti. The post-cultivation liquid appeared to be a more productive source of lipases and proteases than mycelium.

INTRODUCTION

The growth of the mould *P.roqueforti* in fissured Roquefort cheese body is necessary for a sufficiently deep degradation of fat and protein, thus ensuring formation of flavour characteristics, as desired for such and related types of cheese. Flavour characteristics of Camembert and Brie cheese are formed due to the surface growth of the mould *P.candidum* which is considered to be a species identical with *P.caseicolum*, or due to the growth of the mould *P.camemberti.*

The rate and depth of changes which occur in protein and fat of mould cheeses and their flavour qualities are chiefly determined by the lipolytic and proteolytic activity of the said mould species. Mould strains which are used for cheese manufacture may be considered as a biological source to obtain lipolytic and proteolytic preparations to be utilized in both cheese manufacture and other branches of the food industry. Our previous inwestigations [4] showed that lipases which are producted by *P.roqueforti* and *P.candium* are characterized by a hight activity in rela-

tion to lower fatty acids of milk fat which should be considered as an advantageous characteristic from the point of view of modifying cheese flavour characteristics and especially for the manufacture of Italian type piquant cheese. Numerous investigations [2, 3, 5, 6, 8, 11, 13] showed that the mould strains which were used in cheese manufacture, had very differentiated lipolytic and proteolytic activities. Considering the various research and cultivation procedures used, various definitions of proteolytic activity units and the fact that most investigations were limited to one species only, a comparison of the results involves considerable difficulties. In order to improve the quality of mould cultures which are used in cheese manufacture and the selection of mould strains which would be suitable for the production of enzyme preparations, an attempt was made to investigate the abilities of moulds, *P.roqueforti*, *P.candidum* and *P.camemberti*, to produce extracellular lipases and proteases in identical cultivation conditions.

MATERIAL AND METHODS

BIOLOGICAL MATERIAL

Thirty four strains of *P.roqueforti*, 18 strains of *P.candidum* and 13 strains of *P.camemberti*, all belonging to the collection of the Production Laboratory of Dairy Preparations at Olsztyn, were used for the investigations. Strains were stored on malt agar slants at 4°C. In order to obtain an inoculum, mould spores were transferred on fresh prepared slant which was then incubated for 14 days, at 21°C. Subsequently, the spores were removed from slants by rinsing with 200 ml saline which was then used to inoculate the medium in Roux bottels. The bottles were incubated for 18 days, at 21°C. Spore counts in physiological liquid, used as inoculum, were determined by plating.

CULTIVATION METHOD AND CONDITIONS

In order to determine the lipolytic and proteolytic activities of strains studied, moulds were cultivated on a medium which contained 70% rennet whey, 29% malt wort and 1% peptone. The pH value of the medium was adjusted to 6.0; 0.2% of agar was added to the medium. Our previous investigations [2, 13] showed that the above madium provided a good growth of moulds as cultivated by the surface method and the agar added in our experiment, increased the viscosity of the medium, thus favoring a more equal growth of mould on the surface; 200 ml of the medium were transferred into Roux bottles and sufficient amount of inoculum was added to obtain a final spore concentration of 1×10^5/ml. The culture was propagated for 12 days, at 25°C.

ENZYME MATERIAL

The source of extracellular enzymes was a post-cultivation liquid which was obtained by removing mycelium, followed by filtration through Whatman paper 40. The source of intracellular enzymes was a mycelium which was washed three times with water, mixed with water in a ratio of 1:1 and then desintegrated using a Biotec Desintegrator (Sweden). The frozen material was pressed through the desintegrator valve under a pressure of 500 kG/cm^2.

DETERMINATION OF LIPOLYTIC ACTIVITY

The lipolytic activity was determined as related to milk fat, by titrating free fatty acids which were liberated from milk fat emulsion at a constant pH. The 10% emulsion of milk fat was prepared in 5% arabic gum, as described by Parry et al. [9], 100 ml of emulsion was supplemented with 10 ml 0.075 M $CaCl_2$, 10 ml 0.3 M NaCl and 300 mg taurocholate, according to San Clemente and Vadehra [12]; 1 ml of enzyme material was added to 9 ml of a thus prepared substrate. Measurement was carried out at 37°C and pH = 7.2 during 5 minutes. Free fatty acids were titrated with 0.1n NaOH using a pH-stat, composed of the following Radiometer devices: PHM62 — pHmeter, TTT-60 titrator, ABU-13 autoburette and REC61/REA160 servograph.

Lipase contents were expressed in lipolytic activity units (LAu) assuming that one unit corresponds to 1 μmol of free fatty acids (FFA) as liberated in one hour from 9 ml of substrate by 1 ml of post-cultivation medium or 1 ml of desintegrated mycelium solution. The lipolytic activity was expressed in LAu/ml of post-cultivation liquid or LAu/100 g of dry mycelium.

DETERMINATION OF PROTEOLYTIC ACTIVITY

The proteolytic activity was determined by Anson procedure [1]. The substrate, 1% isoelectric casein solution (Fluka Company) was obtained by disssolving casein in 0.15 M phosphate buffer. Proteolytic activity was parallelly determined at pH 5.5 and 7.0 by incubating 9 ml of substrate and 1 ml of enzyme material for 2 hours at 37°C. Upon treating with an equal volume 12% trichloracetic acid, the filtrate obtained was treated with Folin reagent and then the optical density was measured by spectrophotometry at the wave-length of 650 nm.

The proteolytic activity was expressed in proteolytic activity units (PAu), assuming that one such unit corresponds to an increase of optical density by 0.01 during the incubation of the substrate with the enzyme material for 1 hour. The activity was expressed in PAu/ml of nutrient

medium or PAu/100 mg of d.m.mycelium. After 12 days of cultivation, dry matter of mycelium and pH of nutrient medium were determined, as well as spore counts, as produced by moulds on 1 dcm² of the medium.

RESULTS AND DISCUSSION

The strains of each mould species studied were arbitrarily classed into 4 groups, in respect to lipolytic and proteolytic activity of their extracellular enzymes. The lipolytic or proteolytic activity of low-active strains did not exceed 5 u./ml, that of middle active strains was between 5-15 u./ml, that of high-active strains 15 to 25 u./ml, and that of very high-active strains exceeded 25 u./ml (Table 1, 2). The same classification was used when assaying lipolytic and proteolytic activity of intracellular enzymes. In individual groups, the respective activity was: less than 10, 10-25, 25-50 and more than 50 u./100 g (Table 3, 4). In the group of 13 *P.camemberti* strains no strains showing a very high lipolytic activity of extra and intracellular enzymes were found.

The fewest strains with high and very high proteolytic activity were found within *P.roqueforti* species. Niki and coll. [8] suggested that strains of a high lipolytic activity and moderate proteolytic activity should be selected for Roquefort cheese manufacture. For the majority of strains of the species studied, a direct relation was observed between the extra and intracellular lipolytic activities. Among the 64 strains studied 46.8% were classified in identical groups in terms of their extra and intracellular lipolytical activity, and no case was noted in which low intracellular activity would be accompanied by a high extracellular one or vice versa. A higher differentiation was found in extra and intracellular proteolytic activities as measured at both 5.5 and 7.0 pH values. Measurements at pH 5.5 and 7.0 were carried out in view of relatively considerable discrepancies in the observations of various authors [6, 7, 8] concerning the optimum for the action of enzymes which are produced by the mould species being the subject of our studies. Within each species, some differences were observed in the proportions between intra and extracellular proteolytic activities as measured at pH 5.5 and 7.0.

The above observations indicate that a non-homogeneous protease system is formed by the moulds studied and that, after 12 days of cultivation, the proportion between slightly acid proteases and the neutral ones is differentiated to a large extent, depending on the mould strain. Proportions between the lipolytic and proteolytic activities, measured at pH 5.5 and 7.0 varied considerably (Table 1, 3) what indicates that many strains may be selected from the population studied showing relatively high lipolytic activity and low proteolytic one or vice versa. They could be suitable for the production of either lipolytic or proteolytic preparations. Lambert and

Table 1. Lipolytic activity of extracellular enzymes of *P. roqueforti*, *P. candidum* and *P. camemberti* strains

Enzymatic activity		Lipolytic activity LAu · /1 ml medium				Spore counts on 1 dm² of area*)	Amount of dry mycelium g/l of medium*)	Ratio of lipolytic (LA) to proteolytic (PA) activity at different pH*)	
		range	average of the group	number of strains	percentage of strains			$\frac{LA \cdot 100}{PA \text{ at pH } 5.5}$	$\frac{LA \cdot 100}{PA \text{ at pH } 7.0}$
P. roqueforti	low	0.80- 2.10	1.60	3	8.82	0.70- 3.08	13.50-16.00	60.50-4062.50	25.0 -4652.50
	middle	5.20-14.30	9.94	16	47.05	0.28-10.40	15.14-23.76	1.70-416.00	17.40-57.60
	high	15.50-24.80	18.90	12	35.29	0.10- 8.20	8.74-20.28	1.00-40.50	6.80-10.30
	very high	27.10-31.80	30.03	3	8.82	1.26- 4.35	13.60-18.26	14.70-57.20	17.50-38.00
P. candidum	low	0.20- 4.64	2.56	6	33.30	0.32- 8.60	4.10-18.35	42.50-159.50	185.00- 600.00
	middle	9.20-14.08	11.56	5	27.70	0.88- 3.55	7.70-17.90	22.90-73.90	18.40-89.10
	high	15.10-23.24	18.86	5	27.70	1.20- 8.07	12.10-21.00	15.00-102.60	55.00-102.60
	very high	26.32-30.12	28.22	2	11.10	1.20- 3.40	10.60-18.49	24.90-28.10	21.58-31.15
P. camemberti	low	0.00- 3.20	1.40	4	30.76	0.76- 1.51	14.93-20.13	0.00-1443.75	0.00-937.00
	middle	5.20-14.80	10.50	7	53.84	0.38- 1.76	4.30-19.50	36.50-413.46	122.30-413.00
	high	16.68-19.80	18.54	2	15.38	0.15- 0.76	10.52-18.50	27.50-103.50	22.80-73.70
	very high	—	—	—	—	—	—	—	—

*) range

Table 2. Proteolytic activity PA of extracellular enzymes of strains *P. roqueforti*, *P. candidum* and *P. camemberti*

Enzymatic activity		Proteolytic activity(PAu/ 1 ml of medium)								Spore counts on 1 dm^2 of area at pH 7,0	Amount of dry mycelium g/l of medium	Ratio of proteolytic activity at pH 5,5 to that at pH 7,0
		pH 5,5				pH 7,0						
		range	average of the group	number of strains	percentage of strains	range	average of the group	number of strains	percentage of strains			
P. roqueforti	low	0.20-4.50	2.23	18	52.94	0.50-5.00	2.07	27	79.41	0.28-10.40	11.87-23.76	1.11-2.50
	middle	5.10-12.00	6.54	12	35.29	6.20-12.10	7.44	5	14.70	0.84-8.20	8.74-18.86	1.00-1.26
	high	18.20-20.00	19.10	2	5.88	—	—	—	—	—	—	—
	very high	30.00-32.50	31.25	2	5.88	28.00-36.50	32.25	2	5.88	0.10-0.70	13.82-16.00	0.93-1.12
P. candidum	low	2.50-3.50	3.16	5	27.80	1.00-2.60	1.80	3	16.67	0.32-8.07	17.53-21.00	0.40-0.74
	middle	6.80-12.00	8.66	9	50.00	6.50-14.60	10.64	10	35.56	0.75-8.60	7.20-18.49	0.93-1.22
	high	15.50-22.40	18.95	2	11.11	15.50-21.80	18.53	3	16.67	1.20-3.55	17.20-19.09	0.97-1.00
	very high	31.50-50.50	41.00	2	11.11	31.50-32.30	31.90	2	11.11	1.05-2.59	4.10-4.96	0.64-1.00
P. camemberti	low	2.80-4.60	3.67	3	23.08	3.40-3.80	3.60	2	15.38	0.38-0.76	10.52-19.50	0.83-1.21
	middle	5.40-12.50	7.82	4	30.76	6.20-14.60	11.30	6	46.15	0.15-2.70	12.40-18.50	1.15-1.17
	high	18.20-22.40	20.98	5	38.46	18.10-21.50	20.36	3	23.07	0.49-1.76	4.30-17.85	0.96-0.99
	very high	46.20	46.20	1	7.69	25.60-3.00	27.80	2	15.38	1.06-1.24	13.70-20.13	0.55-0.65

Table 3. Lipolytic activity of intracellular enzymes of *P. roqueforti*, *P. candidum* and *P. camemberti* strains

Enzymatic activity		Lipolytic activity (LAu.)100 mg of dry mycelium				Ratio of lipolytic (LA) to proteolytic (PA) activitity at different pH		Spore counts on 1 dcm² of area $\times 10^9$	Amount of dry mycelium g/l of medium
		range	average of the group	number of strains	percentage od strains	$\frac{LA \cdot 100}{PA \text{ at pH } 5,5}$	$\frac{LA \cdot 100}{PA \text{ at pH } 7}$		
P. roqueforti	low	2.80-9.90	6.10	7	20.58	59.40-373.70	36.20-373.70	0.28-10.40	13.50-23.76
	middle	15.10-24.30	19.21	9	26.47	34.70-150.90	39.20-252.30	0.63-5.61	9.44-20.09
	high	25.50-45.50	35.80	14	41.17	22.80-200.00	27.80-212.20	0.10-8.20	8.74-18.85
	very high	50.20-85.60	61.97	4	11.76	20.30-62.70	37.10-82.60	1.12-2.54	13.60-18.26
P. candidum	low	8.90-9.83	9.36	2	11.11	340.80-1227.00	287.20-1666.00	0.75-1.05	4.96-7.20
	middle	12.30-24.00	17.01	7	38.88	0.00-632.20	0.00-702.10	0.88-8.60	4.10-19.09
	high	28.80-37.90	31.90	4	22.22	4.00-167.20	2.63-180.40	1.50-8.07	11.40-21.00
	very high	51.80-67.05	58.62	5	27.77	5.78-112.20	16.40-137.00	0.32-1.64	12.10-18.49
P. camemberti	low	0.10-6.80	1.77	5	30.76	636.50-3640.00	740.40-3960.00	0.38-1.76	4.30-20.13
	middle	12.00-24.50	18.76	5	38.46	19.18-191.10	47.13-235.00	0.49-2.70	10.52-17.40
	high	26.70-38.06	30.52	3	23.07	19.90-124.60	56.50-107.70	0.15-1.10	12.40-18.50
	very high	—	—	—	—	—	—	—	—

Table 4. Proteolytic activity of intracellular enzymes of *P. roqueforti*, *P. candidum* and *P. camemberti* strains

Enzymatic activity		Proteolytic activity (PAu.)100 mg of dry mycelium								Ratio of proteolytic activity at pH 5.5 to the one at pH 7.0
		pH 5.5				pH 7.0				
		range	average of the group	number of strains	percentage of strains	range	average of the group	number of strains	percentage of strains	
P. roqueforti	low	6.40-9.70	8.16	5	14.70	6.52-9.60	8.06	2	5.88	0.99-1.02
	middle	10.74-24.10	18.71	19	55.88	11.40-24.08	19.03	16	47.06	0.99-1.06
	high	25.20-43.20	30.13	10	29.41	26.20-46.20	34.01	13	38.23	1.04-1.07
	very high	—	—	—	—	51.20-60.00	56.80	3	8.82	—
P. candidum	low	3.60-5.75	4.67	2	11.11	4.20-8.60	6.40	2	11.11	1.17-1.50
	middle	12.10-21.50	17.76	8	44.44	13.50-23.10	19.07	8	44.44	1.07-1.12
	high	25.30-44.30	33.73	6	33.33	28.20-39.50	31.65	4	22.22	0.89-1.11
	very high	52.30-58.10	55.25	2	11.11	50.50-65.50	60.17	4	22.22	0.96-1.13
P. camemberti	low	7.60	7.60	1	7.69	—	—	—	—	—
	middle	12.00-21.50	17.31	7	53.85	11.50-21.80	16.96	6	46.15	0.96-1.01
	high	31.50-38.20	34.16	—	38.46	26.20-47.50	34.34	7	53.84	0.83-1.24
	very high	—	—	—	—	—	—	—	—	—

Lenoir [5] suggested the existence of direct proportionality between the activities of extracellular proteases and lipases of *P.caseicolum*. Opposite relationships were observed by Niki et al. [8] when studying the strains of *P.roqueforti*. Our findings do not support the assumption that there is a direct relationship between extra and intracellular proteolytic and lipolytic activities. In general it may be stated that the strain group of *P.roqueforti* and *P.candidum* was represented by strains showing a higher lipolytic activity than that of the *P.camemberti* strain group.

Prokš and Cingrašova [10] investigated several strains and concluded that *P.caseicolum* was more lipolytically active than *P.camemberti* species. This observation has been supported by our own findings. When comparing the activity of 1 ml of medium with the lipolytic activity of the same volume of mycelium, the postcultivation medium was found to be a much more productive source of lipases than mycelium. On the basis of that comparison it can be stated that for 70% of strains of *P.roqueforti* and 100% of *P.candidum* the post cultivation liquid is twice as productive a source of lipases as mycelium. When analysing the above relationships between extra and intracellular proteolytic activities, it may be concluded that in the case of 100% strains with very high proteolytic activity the post-cultivation medium is a better source of proteases than mycelium, as measured at both pH 5.5 and pH 7.0.

Previous investigations showed the large differentiation of mould strains, as used in cheese manufacture, in their ability of protein and fat degradation. These differences were observed when analysing the level of lipases and proteases in post cultivation liquids [5, 6, 8, 13] or when determining the degradation extent of protein and fat in cheeses [2, 11]. The studies of Niki et al. [8], Habaj et al. [2], Prokš et al. [1] on *P.roqueforti* strains, and those of Lambert and Lenoir [3], concering *P.caseicolum* strains, showed within either of those species it is possible to find strains which would differ about 10-times from one another in their extracellular lipolytic activity. Within three mould species of our collection, strains may also be found, which differ from each other to a similar extent in both their proteolytic and lipolytic activity. A lower differentiation than that shown by us, i.e. 5-times only, was found by Lenoir and Choisy [6] who studied the proteolytic activity of *P.caseicolum* strains.

The results of studies, as shown in Tables [1, 2] indicate that there is no relationship between proteolytic or lipolytic activity, the amount of mycelium biomass or the amount of spores produced. On the basis of these results it may be stated that all three species studied are comparable in terms of the amount of mycelium biomass or spores produced, although under the cultivation conditions used individual strains differed within each species about two or more times, in the amounts of mycelium or spores produced.

The nutrient medium of the majority of strains studied underwent alkalization after 12 days, the pH values of the medium varying in the period of cultivation from the initial value of pH 6 up to 7.2-8.5, depending on the strain.

CONCLUSIONS

1. The strains *P.roqueforti*, *P.candidum* and *P.camemberti* differ from each other, within each species, by up to 10-times in the lipolytic or proteolytic activities of both their extracellular and intracellular enzymes. The *P.roqueforti* and *P.candidum* species are represented by strains showing higher lipolytic activity as compared to that of *P.camemberti* species.

2. From the collection of three mould species studied, strains were selected having differentiated enzyme activities which could be used to obtain lipolytic or proteolytic preparations. The post-cultivation liquid appeared to be a more productive source of lipases and proteases than mycelium.

3. No relationship was found between the levels of lipolytic or proteolytic activities, as well as between the activities of lipases and proteases, and, on the other hand, the amount of mycelium biomass and spore contents produced by *P.roqueforti*, *P.candidum* and *P.camemberti* strains.

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Manuscript received: June, 1979
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AKTYWNOŚĆ LIPOLITYCZNA I PROTEOLITYCZNA SZCZEPÓW PLEŚNI P. ROQUEFORTI, P. CANDIDUM I P. CAMEMBERTI

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S t r e s z c z e n i e

Badano aktywność lipolityczną i proteolityczną enzymów zewnątrz i wewnętrzkomórkowych 34 szczepów P. roqueforti, 18 szczepów P. candidum i 13 szczepów P. camemberti. Aktywność lipolityczną określano w stosunku do emulsji tłuszczu mlecznego, a aktywność proteolityczną w stosunku do kazeiny izoelektrycznej. Źródłem enzymów był płyn pohodowlany i grzybnia po powierzchniowej hodowli pleśni prowadzonej w czasie 12 dni. Szczepy każdego gatunku pleśni podzielono umownie na 4 grupy pod względem aktywności enzymów lipolitycznych lub proteolitycznych. W skrajnych przypadkach grupy różniły się ponad 10-krotnie aktywnością lipolityczną lub proteolityczną. Szczepy P. roqueforti i P. candidum były bardziej aktywne lipolitycznie w porównaniu ze szczepami P. camemberti. Biorąc pod uwagę proporcje pomiędzy aktywnością lipolityczną i proteolityczną pleśni z badanej kolekcji można wyselekcjonować szczepy nadające się zarówno do pozyskiwania preparatów proteolitycznych jak i lipolitycznych. Nie stwierdzono zależności pomiędzy aktywnością lipolityczną a proteolityczną oraz pomiędzy aktywnością enzymatyczną a ilością biomasy grzybni i ilością zarodników pleśni. Płyn pohodowlany bardzo wysoko aktywnych lipolitycznie szczepów jest wydajniejszym źródłem lipaz i proteaz niż grzybnia.