

## EXTRACELLULAR PROTEINASES IN NATURAL ISOLATES OF STAPHYLOCOCCI

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*Biochemical characteristics of proteinases from natural isolates of Staphylococcus sp. F22, F86, M104, S2007 and S2105 have been studied. It was found that these proteinases have relatively low molecular masses (from 20 to 32 kDa), and that they are released from the cell envelope in to the growth medium. Their temperature optima are between 30 and 37°C and their pH optima range from 6,5 to 8. Copper ions inhibit their activity, but the presence of calcium ions stimulates the activity of proteinases from isolates F22, M104 and S2007. Beside casein fractions, they also hydrolyze heterologous protein substrates, such as BSA and gelatin. Experiments with specific proteinase inhibitors revealed that proteinases from isolates F22 and M104 belong to the serine group of proteinases, S2007 and S2105 proteinases were classified as metalloproteinases. Type of F86 proteinase in these experiments could not be clearly determined.*

*Key words: casein, inhibitors, proteinases, Staphylococcus*

### INTRODUCTION

Staphylococci are Gram-positive, nonmotile, aerobic or facultatively anaerobic bacteria and represent the largest group in the *Micrococcaceae* family. Their natural habitats are the skin and mucous membranes of mammals, but they can also be isolated from samples of raw milk and fermented milk products, meat products, soil, air and natural waters (Kloos and Schleifer, 1986, Bautista *et al*, 1986). Although some important pathogens belong to the genus *Staphylococcus* (*Staphylococcus aureus*, *S. haemolyticus*), many species from this group are saprophytic microorganisms and some of them (*S. carnosus*) are used as a starter cultures for the production of fermented foods (Kloos and Schleifer, 1986). Staphylococci can produce a wide variety of extracellular enzymes. Some of them, like for example hemolysins and heat-resistant nuclease are associated with pathogenicity. Other enzymes, produced by non-pathogenic strains, include lipases, glycosidases and different types of extracellular proteinases.

The proteolytic activity of staphylococci and micrococci has been intensively studied in order to elucidate its role in the production of fermented foods and in processes caused by food-borne pathogens or clinical isolates which belong to this group of bacteria. In previous studies, it has been shown that staphylococci synthesize extracellular proteinases that belong to different classes.

Among them, metalloproteinases are the most common proteolytic enzymes, since they are produced by *S. epidermidis* (Teufel and Gotz, 1993), *S. hyicus* subsp. *hyicus* (Ayora and Gotz, 1994), *S. aureus* (Banbula *et al.*, 1998), and by *S. caseolyticus*, previously classified as *Micrococcus caseolyticus* (Desmazeaud and Hermier, 1968). The enzymes from the classes studied so far have neutral pH optima, molecular masses ranging between 30 and 40 kDa and for the catalytic activity require the presence of zinc in the active site. Aureolysin, a zinc-dependent metalloproteinase from *S. aureus*, exhibits its activity against several proteins which are important for the host, suggesting the involvement of this enzyme in the pathology of staphylococcal infections (Banbula *et al.*, 1998). The *aur* gene, encoding aureolysin, was analyzed in natural isolates of *S. aureus* from different domestic animals. The PCR-RFLP analysis indicated that the *aur* gene is highly conserved among *S. aureus* isolates from different hosts (Takeuchi *et al.*, 2002).

Among extracellular serine proteinases, the V 8 protease from *S. aureus* has been the most intensively studied. The V 8, or Ssp serine protease, is the product of the *sspA* gene, and contributes to the growth and survival of *S. aureus* during the infection (Rice *et al.*, 2001). Another gene, *sspB*, is located within the same operon and encodes 40,6 kDa cysteine proteinase, which is processed by the V 8 protease. A similar operon, containing the genes for extracellular serine and cysteine proteinases, was found in *S. epidermidis* (Dubin *et al.*, 2001). The serine proteinase from natural isolate of *S. warneri* was also homologue to V 8 protease on the amino acid sequence level, and exhibited the same substrate specificity, as it selectively cleaved the carbonyl side of glutamic acid residues in  $\beta$ -casein (Yokoi *et al.*, 2001). Another strain of *S. aureus*, isolated from a diseased chicken, produces an extracellular cysteine proteinase which is different from SspB proteinase, with a molecular mass estimated at 23 kDa (Takeuchi *et al.*, 1990).

Apart from the role in staphylococcal infections, the proteolytic activity of non-pathogenic staphylococci is very important in the manufacturing process of fermented foods. It was shown that *S. caseolyticus*, as well as several strains of micrococci, significantly contributes to proteolysis during cheese ripening (Bhowmik and Marth, 1988). *Staphylococcus* sp. M104, the strain analyzed in this work, was successfully used as a component of the starter culture for cheese (Stević *et al.*, 1973) and production of fermented sausages (Šutić and Joksimović, 1973). It was also shown that the strain M104 produces an extracellular proteinase with a strong proteolytic activity towards casein and that in mixed cultures stimulates the growth of lactic acid bacteria (Obradović, 1983). The same effect was observed with the strain *S. caseolyticus* M96 (Desmazeaud and Devoyod, 1970). In this work, we describe the biochemical characterization of extracellular proteinases from the strain *Staphylococcus* sp. M104 and other four non-pathogenic natural isolates of staphylococci.

## MATERIALS AND METHODS

### *Bacterial strains and media*

The following strains of staphylococci were used in the experiment: *Staphylococcus* sp. M104 (Laboratory collection, Faculty of Agriculture, Belgrade), *Staphylococcus* sp. F22 (natural isolate from raw milk), *Staphylococcus* sp. F86 (natural isolate from UHT milk), and *Staphylococcus* sp. S2007 and S2105 (natural isolates from silage). The strain *Lactococcus lactis* subsp. *lactis* MG1363 (Gasson, 1983) was used as proteinase-negative control. Strains of staphylococci were grown and maintained in nutrient broth (Torlak, Belgrade), at 30°C in aerobic conditions. Agar plates were prepared by addition of agar (1.5%, wt/vol) to the nutrient broth. *Lactococcus lactis* subsp. *lactis* was grown at 30°C in aerobic conditions in M17 medium (Terzaghi and Sandine, 1975), supplemented with glucose (0.5% wt/vol) to which agar (1.5%, wt/vol) was added to solidify the medium. To test the proteolytic activity, the cells were grown on milk-citrate agar (MCA) plates containing 4.4 % reconstituted non-fat skim milk (RSM), 0.8 % Na<sub>3</sub>-citrate, 0.1% yeast extract, 0.5 % glucose and 1.5% agar (wt/vol). For the screening of strains with proteolytic activity casein agar containing 1,2% of total casein (Sigma Chemie GmbH, Deisenhofen, Germany) and 1.5% agar (wt/vol), dissolved in 100 mM Tris-HCl buffer (pH 7.8) was used.

### *Assay of proteinase activity*

Proteolytic activities of *Staphylococcus* strains were assayed as described (Kojic *et al.*, 1991, Kojic *et al.*, 1995). For enzymatic assays, the strains were grown on MCA plates for 48 h at 30°C prior to cell collection. Collected fresh cells (2 mg; approx. density of 10<sup>9</sup> cells/ml) were resuspended in 100 mM Na-phosphate buffer (pH 7.2). The cell suspension was mixed with the substrate dissolved in the same buffer at a 1:1 volume ratio. As a substrate, we used α<sub>S1</sub>-, β-, and κ-casein fractions, denatured bovine serum albumin (BSA), gelatin and hemoglobin (Sigma Chemie GmbH, Deisenhofen, Germany) (5 mg/ml). After incubation at 30°C cells were pelleted by centrifugation (5 min at 12000xg), the supernatant was removed and samples for SDS-PAGE were prepared. Analysis of substrate hydrolysis was done by SDS-PAGE by loading 15% (wt/vol) acrylamide gel with the prepared samples.

### *Preparation of crude extracts and test of activity*

To obtain the crude proteinase extract the cells were grown on MCA plates for 48 h at 30°C. After incubation, cells were collected, washed twice in 100 mM Na-phosphate buffer (pH 7.2) and two extracts were pooled. Protein concentrations in crude extracts were measured by the method of Lowry *et al.* (1951) with BSA (Sigma Chemie GmbH, Deisenhofen, Germany) as a standard. The substrate (β-casein, 5 mg/ml in 100 mM Na-phosphate buffer pH7.2) was mixed with crude extracts and incubated at 30°C. Samples were taken from the reaction mixture at different time intervals, mixed with the same volume of sample buffer and analyzed by SDS-PAGE.

#### *Determination of pH and temperature optima*

To test the influence of pH conditions on proteinase activity, the reaction mixtures containing cells and  $\beta$ -casein were incubated in either 100 mM Na-phosphate buffer (pH 5.4, 5.7, 6.5 or 7.2) or in 100 mM Tris-HCl buffer (pH 8.0 or 8.7) for 1 hour at 30°C. In order to determine the temperature optimum for proteolytic activity, the reaction mixtures, prepared in 100 mM Na-phosphate buffer (pH 7.2) were incubated at 25, 30, 37, 42, 50 and 60°C for 1 hour. In both cases, the reactions were stopped by addition of 1/2 volume of 50% trichloroacetic acid (TCA) and hydrolytic products were quantified by the method of Lowry *et al.* (1951). Arbitrary unit (AU) of the proteolytic activity was taken as the amount of the enzyme which releases 1  $\mu$ g of TCA soluble products per reaction mixture within 1 hour.

#### *Effect of ions and inhibitors on the proteolytic activity*

The effect of ions and inhibitors on the proteolytic activity was tested with crude proteinase extracts, which were dialyzed against 100 mM Tris-HCl buffer pH 7.2 at 1 : 500 volume ratio for 24 hours at 4°C, with one buffer replacement. After adding ions or inhibitors, the extracts were incubated for 10 min at 30°C prior to addition of substrate ( $\beta$ -casein, 5 mg/ml in 100 mM Na-phosphate buffer pH 7.2) in 1:1 volume ratio. After adding the substrate incubation was continued for 30 min. The reaction was stopped by addition of TCA and TCA-soluble hydrolytic products were determined by the method of Lowry *et al.* (1951). All ions and inhibitors were added at 10 mM final concentrations except phenylmethylsulfonyl fluoride (PMSF) (10 mg/ml), pepstatin (0.2 mM), 1,10-phenanthroline (0.5 mM) and dichloroisocoumarin (DCI) (0.1 mM). All ions used in the experiment were in the chemical form of chlorine salts.

#### *Non-denaturing SDS-PAGE*

SDS-PAGE was performed by a modified procedure of MacFarlane and MacFarlane (1992). Crude proteinase extracts were dissolved in sample buffer (1% SDS, 25% glycerol, 0.06% bromphenol blue and 125 mM Tris-HCl pH 6.8). The samples were run on polyacrylamide gels containing 0.25% (wt/vol) of total casein. After electrophoresis, the gels were renatured for 2 hours at room temperature in the buffer containing 25 mM Tris-HCl (pH 8), 0.5% Tween 80 and 0.5% Triton X-100. Molecular masses of staphylococcal proteinases were determined by Rainbow™ Protein Molecular Weight Standard (Amersham Biosciences, Freiburg, Germany).

## RESULTS

#### *General characteristics of the strains and their proteolytic activity*

The strains analyzed in this work are part of a larger collection of 55 natural isolates of staphylococci with proteolytic activity. The strains were isolated from raw milk, UHT milk and silage by standard microbiological methods. All tested strains were non-hemolytic, coagulase negative and showed strong proteolytic

activity on casein agar plates (Figure 1). Further biochemical characterization of extracellular proteinases was performed for strains *Staphylococcus* sp. F22, F86, S2007, S2105 and for the strain M104, which produces an extracellular proteinase that has been partially characterized (Obradović, 1983). In order to determine the substrate specificity of these enzymes, cells of the analyzed strains were collected after growth on MCA plates and their proteolytic activity was tested against different substrates. The results showed that all five strains efficiently hydrolyzed

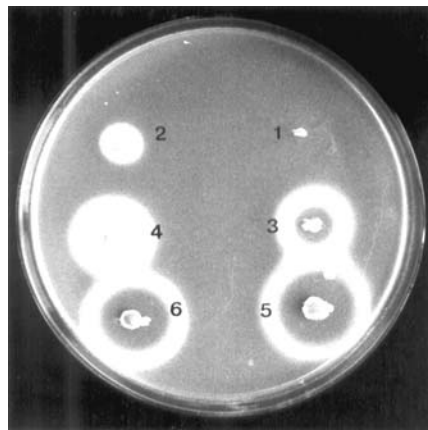


Figure 1. Proteolytic activity of natural isolates of staphylococci on casein agar plates. 1) *Lactococcus lactis* subsp. *lactis* MG1363 (proteinase-negative strain); 2) F22; 3) F86; 4) M104; 5) S2007; 6) S2105.

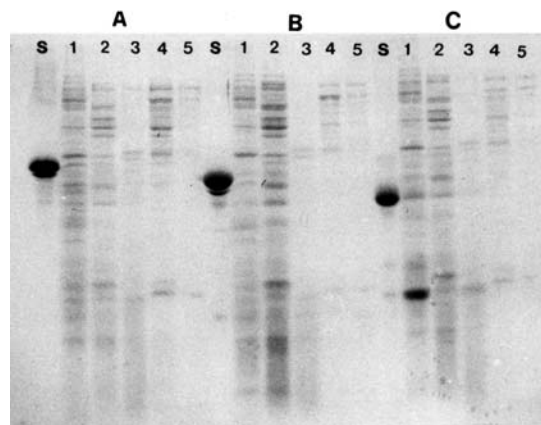


Figure 2. Hydrolysis of casein fractions by whole cells of staphylococci. A)  $\alpha_{S1}$ -casein; B)  $\beta$ -casein; C)  $\kappa$ -casein; Lane s: starting substrate; Lane 1: F22; Lane 2: F86; Lane 3: M104; Lane 4: S2007; Lane 5: S2105. The cells were incubated for 30 min. with  $\alpha_{S1}$ - and  $\beta$ -casein, and for 1 hour with  $\kappa$ -casein.

casein fractions,  $\alpha_{S1}$ -,  $\beta$ - and  $\kappa$ -casein (Figure 2). The activity of the proteinases was also tested against denatured hemoglobin, BSA and gelatin. Strains F86, S2007 and S2105 were able to hydrolyze gelatin after 5 hours of incubation at 30°C. Under the same conditions of hydrolysis, proteinases from all strains (except F22) partially hydrolyzed BSA, but it was not possible to detect any proteolytic degradation when hemoglobin was used as a substrate (data not shown).

The activity of crude proteinase extracts was analyzed using  $\beta$ -casein as a substrate. In this experiment, protein concentrations in crude proteinase extracts were 3 mg/ml for each strain, and the enzyme/substrate ratio was 1 : 1 for the strain F22, 1 : 3 for the strain M104 and 1 : 4 for the remaining strains. The analysis of the kinetics of  $\beta$ -casein degradation (Figure 3) showed different patterns of substrate hydrolysis. We present the results for strains M104, F86 and S2007, since the obtained  $\beta$ -casein hydrolysis pattern for the strain F22 was very similar to the pattern of strain M104 and there was also a great similarity of substrate hydrolysis patterns between the strains S 2105 and S 2007.

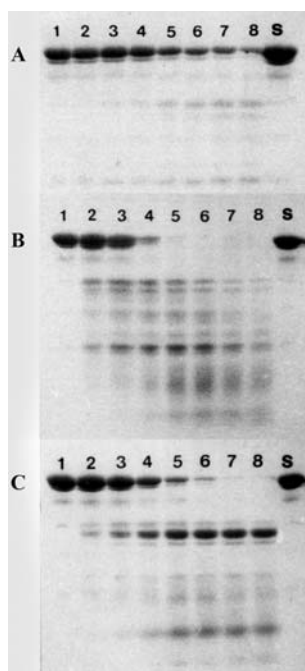


Figure 3. Comparative kinetics of  $\beta$ -casein degradation by crude proteinase extracts of *Staphylococcus* sp. A) M104; B) F86; C) S2007. The samples were taken from the reaction mixture at: Lane 1, 0 min; Lane 2, 5 min; Lane 3, 10 min; Lane 4, 30 min; Lane 5, 60 min; Lane 6, 90 min; Lane 7, 120 min; Lane 8, 180 min. Lane s: starting substrate

*Determination of pH and temperature optima and the effect of ions and inhibitors on the proteolytic activity*

The optimal pH and temperature for hydrolysis of the substrate were determined for all five strains. The obtained values of pH optima were 6.5 for the strain M104, 7.2 for strains F22 and F86, and 8.0 for strains S2007 and S2105. The optimal temperature for the proteolytic activity was 37°C for all strains except for the strain F22, which most efficiently hydrolyzed  $\beta$ -casein at 30°C and generally showed the lowest proteolytic activity.

The effect of mono- or divalent cations on the proteolytic activity was tested by using dialyzed crude proteinase extracts. The results showed that  $\text{Na}^+$  and  $\text{K}^+$  ions did not have any influence on the proteolytic activity, but  $\text{Ca}^{++}$  exhibited a stimulatory effect on proteinases from strains F22, M104 and S2007 (Table 1). The  $\text{Cu}^{++}$  ions inhibited the activity of all tested proteinases, while the  $\text{Zn}^{++}$  ions showed only partial inhibition of proteolytic activity in strains M104, S2007 and S2105. EGTA, EDTA and particularly 1,10-phenanthroline inhibited the activity of proteinases from strains S2007 and S2105, suggesting that these two enzymes belong to the class of metalloproteinases. On the other hand, the addition of specific serine proteinase inhibitors, PMSF and DCI, completely inhibited the activity of proteinases from strains F22 and M104 (Table 1), showing that they belong to the serine proteinases class. Pepstatin and iodoacetate, specific inhibitors of aspartic proteinases and cysteine proteinases, did not show any inhibitory effect on the activity of tested staphylococcal proteinases.

Table 1. Relative proteolytic activity (%) of crude extracts of staphylococcal proteinases in the presence of ions and inhibitors. (The control row represents the activity of a particular proteinase extracts without added ions or inhibitors)

	STRAIN				
	F22	F86	M104	S2007	S2105
Control	100	100	100	100	100
$\text{Zn}^{++}$	103	83	69	77	67
$\text{Ca}^{++}$	134	97	123	111	99
$\text{Cu}^{++}$	21	32	26	45	52
$\text{Na}^+$	104	96	97	107	97
$\text{K}^+$	91	102	105	101	92
EDTA	99	88	95	47	52
EGTA	102	98	101	66	70
PMSF	0	36	4	39	49
PEP	98	103	96	94	101
IAC	100	92	95	105	99
1,10 P	96	94	82	33	37
DCI	0	80	0	92	88

PMSF – phenylmethylsulfonyl fluoride; PEP – pepstatin; IAC – iodoacetate;  
1,10 P -1,10 – phenanthroline; DCI – dichloroisocoumarin

#### *Size determination of staphylococcal proteinases*

Molecular masses of staphylococcal extracellular proteinases were determined by using modified SDS-PAGE performed under non-denaturing conditions and with the separating gel containing casein as a substrate. Crude proteinase extracts, obtained by washing the cells, were run on the gel and the zones of casein hydrolysis appeared at the positions corresponding to the active forms of the enzymes. According to these results, the approximate molecular mass of proteinases was 20 kDa for strains F86 and S2105, 22 kDa for the strain S2007 and 32 kDa for the strain M104 (Figure 4). In each case, only one zone of substrate hydrolysis was observed. Among the tested strains, the crude proteinase extract from the strain F22 was the only one which did not give any visible casein hydrolysis under these experimental conditions, most likely because of its lower proteolytic activity.

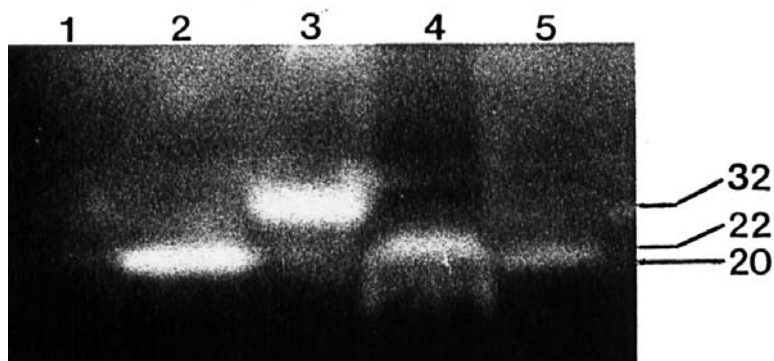


Figure 4. Molecular size determination of staphylococcal extracellular proteinases. Lane 1: F22; Lane 2: F86; Lane 3: M104; Lane 4: S2007; Lane 5: S2105. The approximate molecular masses, corresponding to the substrate hydrolysis zones, are 20 kDa for strains F86 and S2105, 22 kDa for the strain S2007 and 32 kDa for the strain M104

#### DISCUSSION

Extracellular proteinases are produced by many species of Gram-positive and Gram-negative bacteria. Apart from pathogenic species, where they contribute to the virulence of a particular strain during bacterial infections, proteinases and other extracellular enzymes are also produced by saprophytic bacteria which are normal microflora of the soil, water and air. They can be found in different samples of plant and animal origin. In this work, we described the biochemical characterization of extracellular proteinases from non-pathogenic natural isolates of staphylococci. The proteolytic activity of the analyzed strains was tested against different substrates. Proteinases from all strains exhibited high efficiency in the degradation of casein fractions (Figure 2), whereas the gelatin



was hydrolyzed by proteinases from strains F86, S2007 and S2105. Partial hydrolysis of BSA was observed in all strains, except with the proteinase from strain F22, which, in comparison to other strains, showed the lowest level of proteolytic activity. The estimated molecular mass of the tested proteinases was from 20 to 32 kDa (Figure 4). This range is within the values of previously characterized enzymes from staphylococci and micrococci (Desmazeaud and Hermier, 1968, Prasad *et al.*, 1986, Banbula *et al.*, 1998, Yokoi *et al.*, 2001). The analysis by non-denaturing SDS-PAGE also suggested that each natural isolate produces only one active form of the enzyme. In other bacterial species, like for example *Clostridium bif fermentans*, one strain can synthesize many active forms of extracellular proteinases (MacFarlane and MacFarlane, 1992). The experiments with specific proteinase inhibitors (Table 1) showed that proteinases from strains S2007 and S2105 most likely belong to the class of zinc-dependent metalloproteinases, particularly because they are inhibited by 1,10-phenanthroline, which is a highly specific inhibitor. On the other hand, F22 and M104 proteinases obviously belong to the serine class, since they are completely inhibited by PMSF and DCI, which are serine proteinase inhibitors. The proteinase from the strain F86 was only partially inhibited in the presence of specific inhibitors, so on the basis of these results it can not be placed in a particular class. This enzyme could be similar to the V 8 protease from *S. aureus*, which is resistant to all proteinase inhibitors (Beynon and Bond, 1994). According to the results obtained in experiments with specific proteinase inhibitors, neither of the tested strains did not produce proteinases from the aspartic or cysteine class, which are, less common in bacteria than serine proteinases and metalloproteinases (Barrett and Rawlings, 1991).

Bacteria from the family *Micrococcaceae* are the major component of non-starter microflora in fermented foods. Due to their proteolytic activity, they play an important role in the process of cheese ripening (Prasad *et al.*, 1986, Bhowmik and Marth, 1988) and have been used as starter cultures in cheese manufacturing (Bottazi and Battistotti, 1966), as well as in fermented meat products (Fischer and Schleifer, 1980). Also, preparations of microbial proteinases were used during cheesemaking in order to accelerate the ripening process (Law and Wigmore, 1982). Characterization of extracellular proteinases presented in this work could be useful in further experiments in order to design new starter cultures, particularly because the strain *Staphylococcus* sp. M104 was, already used for this purpose.

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## EKSTRACELULARNE PROTEINAZE PRIRODNIH IZOLATA STAFILOKOKA

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### SADRŽAJ

U ovom radu su detaljnije biohemijski okarakterisane proteinaze prirodnih izolata *Staphylococcus* sp. F22, F86, M104, S2007 i S2105. Utvrđeno je da se radi o proteinazama relativno male molekulske mase (od 20 do 32 kDa) koje spadaju u prave ekstracelularne enzime, pošto se sa površine ćelije odvajaju u medijum za rast. Temperaturni optimumi ovih proteinaza se kreću od 30 do 37°C, a pH optimumi u opsegu od 6,5 do 8. Joni bakra inhibiraju njihovu aktivnost, dok joni kalcijuma stimulišu aktivnost proteinaza iz izolata F22, M104 i S2007. Pored kazeinskih frakcija, proteinaze stafilokoka hidrolizuju i druge proteinske supstrate, kao što su želatin i BSA. U eksperimentima sa proteinaznim inhibitorima utvrđeno je da proteinaze izolata F22 i M104 pripadaju serinskoj klasi, proteinaze S2007 i S2105 klasi metaloproteinaza, dok proteinazu izolata F86 na ovaj način nije bilo moguće precizno klasifikovati.