PROTEASE ACTIVITIES OF SEMI-PURIFIED *Pseudomonas fluorescens* IN PROTEIN DEGRADATION OF PASTEURIZED MILK AT STORAGE

Tatik Khusniati[1] and **Irma Normalia**[2]
[1]Microbiology Division, Research Center for Biology, Indonesian Institute of Sciences
Cibinong Science Center, Jl Raya Jakarta-Bogor Km 46, Cibinong 16911,
Phone: (62-21-8765067); Facs: (62-21-8765062); Email: tatikkhusni@yahoo.com
[2]Chemistry Department, Bogor Agricultural University

ABSTRACT

Protein on stored milks spoiled due to protease activities of Pseudomonas fluorescens. To know protease effect on milks, protease activities of semi-purified P. fluorescens on protein degradation in stored pasteurized milks were detected. Protease was semi-purified by ethanol 70%. Protease activities were detected by modified Lowry method, protein degradation by formol titration, and protein content by Kjeldahl method. Milk storage' times were conducted on 0 day (4 days before expired date) up to 12 days (8 days after expired date). The results show that the longer the storage times the higher protease activities and protein degradation of milks. At storage 12 days, protease activities on control were 0.2556 Unit/mL (skim) and 0.2116 Unit/mL (whole), and on inoculated milk (crude) was 2.2044 Unit/mL (skim) and 1.5378 Unit/mL (whole); while on inoculated milk (semi-purified) was 3.5355 Unit/mL (skim) and 1.6778 Unit/mL (whole), respectively. The decrease of inoculated milk' homogeneous were faster than that of control. Protein degradation on control, inoculated skim milks (crude and semi-purified) on 12 days were 4.48%, 7.28% and 7.62%, while that on whole milks were 3.81%, 7.28% and 6.04%, respectively. Based on protease, protein degradation and homogeneous, it can be concluded that skim milk spoiled faster than whole milk.

Key words*: pasteurized milk, protease, Pseudomonas fluorescens, skim, storage*

INTRODUCTION

Protein of pasteurised milks spoiled after use by date, at refrigerated temperatures due to the protease activities of psychrotrophic bacteria, especially *Pseudomonas* spp. The main species of *Pseudomonas* spp. spoiled protein of pasteurized milks was *Pseudomonas fluorescens* (Chandler *et al*., 1990; Deeth *et al*., 2002; Khusniati, 2005).

It has been reported that protein of pasteurized milks at temperature 72^{o} C 15' to 88^{o} C 15' in carton package stored at 4.5 and 7° C spoiled due to the protease activities of psychrotrophic bacteria, especially *Pseudomonas* sp., and the average shelf life of whole milks stored at 4.5 and 7° C were around 7 days in western area (Bishop and White, 1986). However, Heo (1989) examined commercial milk samples at temperature 72^{o} C 15' to 88^{o} C 15' in carton package held at 7.2° C and found that after 10 days of storage, 91% of whole milks with protein as the main nutrition were acceptable, whereas after 14 days, 82% of whole milks were still acceptable, with the only 18% weren't acceptabbe.

The spoilage of protein in pasteurized milks due to the protease activities of psychrotrophic bacteria may resulted in the microbial and chemical changes of the milks' protein and the volatile compounds of the milks (Reinheimer *et al.*, 1993). The biochemical changes of milks' protein at spoilage may resulted from the protease activities (Sorhaug and Stepaniak, 1997), which degraded protein in milks (Hsu, 1984; Janzen *et al*., 1982).

The protease activities of *Pseudomonas fluorescens* which degraded protein on pasteurized milks at storage hasn't been reported yet. It has been known that the microbial protease activities can be semi-purified by using chemical method. This papers reports protease activities of semi-purified *Pseudomonas fluorescens* on protein degradation in pasteurized milks at storage

METHODS

Pseudomonas fluorescens suspension

The slant culture of *Ps. fluorescens* was poured into 10 mL liquid of sterilized Luria Bertani media, and it was incubated and shaked overnight. The incubated culture was measured up to OD 0.5 on λ540 nm.

Protease production

The amount of 2% inoculum of *Ps. fluorescens* with OD 0.5 was inoculated into the media skim milks (10% skim milks in aquadest and sterilized), incubated at room temperature and shaked for 2-4 days. After shaking,

the liquid was centrifuged on 8400 g for 5 minutes at temperature 4° C. The supernatant found was measured its protease activities and some supernatans were used for semi-purification of protease.

Semi-purification of protease

Protease was semi-purified by ethanol 70%. The 5 erlenmeyers were prepared, and each erlenmeyer was filled with 25 mL protease solution. The protease solution was precipitated gently by adding ethanol up to concentration 70% (v/v) and it was then homogenized at temperature 4^0C. The homogenized solution was kept overnight at refrigerated room, centrifuged on 8400 g at temperature 4° C for 15 minutes. The precipitate found was soluted in 5 mL buffer phosphate 0.01 M with pH 8. The precipitate soluted was measured its protease activities and added into the milks for detection protease activities.

Protease activities

Protease activities were detected by modified Lowry method (Meloan and Pomeranz, 1973). The amount of 1.00 mL buffer phosphate 0.05 M pH 8 and 1 mL protease were poured into the small tube and incubated on 37° C for 5 minutes. The solution was then added 1 mL casein 2% and incubated at the same temperature 37° C for 30 minutes. At the time of 30 minutes, the reaction was stopped by adding 2.00 mL TCA 0.40 M and homogenized by shaking. The precipitate formed was filtered by filter paper. The amount of 1 mL liquid produced was pipetted and added 5 mL Na_2CO_3 0.5 M, homogenized and incubated at 37° C for 5 minutes. The solution was then added 1.00 mL Folin Ciocalteau, and incubated for 30 minutes and measured the value of abosorbance on λ 660 nm. Every treatment was used control with the same procedure, but for control TCA was added before the add of casein. The tyrosin solution was used as standard which it was soluted in HCL 0.1 M at various concentrations. For the detection of sample concentration, it was made standard curve with calculation as follow:

$$\text{Protease activities (U/mL)} = X \times fp \times 1000 \times 1/V \times T \quad \text{.....(1)}$$

X = concentration of enzyme from regresion equation with absorbance value as Y
Fp = factor of dilution
V = volume of enzyme tested
1000 = conversion factor (mM to M)
T = incubation time (30 minutes)

Protein degradation

Protein degradation was detected by formol titration with modification (King, 1978). The 10 mL of milks' samples was poured into 20 mL aquadest, 0.4 ml saturated K-oxalate and 1 mL indicator phenol-phtalein 1%, and it was kept for 2 minutes. The mixed solution was then titrated by NaOH 0.1 N up to the colour to be pink. The pink solution was added 2 ml formaldehide 37% and it was then titrated by the solution of NaOH 0.1 N up to the colour to be rose-apple red. The volume of titration after adding formaldehide was corrected by the titration volume of control and it was used to detect protein concentration on milks.

Protein contents

Protein contents were detected by Kjeldahl method with modification (Meloan and Pomeranz, 1973). The milk samples both with and without inoculated by protease *P. fluorescens* (crude and semi-purified), were firstly prepared by rapidly shaking and warming up to 40° C, and it was then shaked and cooled up to 20° C. The 5 gr aliquot formed was taken and this aliquot was transferred into Kjeldahl flask, and added 0.5 gr of the mix selenium and 10 mL H_2SO_4. The solution in the Kjeldahl flask was heated, up to black colour. After black colour forming, the heating was increased up to ckean green colour formed. The clean solution was added gently 10 mL water and cooled for 15 minutes. The solution was then transferred qualitatively into the measuring flask 100 mL and added aquadest up to the line.

The 10 mL of liquid sample was pipetted and put into distillation flask and added 10 mL NaOH solution 30%. The destilate was poured into erlenmeyer which contain 5 mL H_3BO_3 4% and 2-4 drops mixed indicator (methyl red 0.2% in alcohol and methylene blue 0.2% in alcohol with comparison 2:1), and it was put on condenser which soaking under solution of H_3BO_3 4%. The comparison was conducted up to the clean green colour formed. The condenser tube was washed and the washing water was put on the same erlenmeyer. The distillation result was titrated by solution of HCl 0.02 N up to the colour to be pink. The detection of control was conducted by the same procedure without the add of sample.

The formulae of crude protein concentration:

$$\text{N concentration (\%)} = \frac{(VHCl - Vcontrol) \times N\ HCl \times 14{,}008 \times 10 \times 100\%}{\text{mg sample}} \quad \text{.....(2)}$$

$$\text{Protein concentration (\%): N concentration (\%)} \times 6.38 \text{.....(3)}$$

V HCl = volume HCl 0.02N used for titration sample solution
V control = volume HCl 0.02N used for titration control solution
6.38 = conversion factor of protein concentration on milks
10 = dilution factor 100/10

Protease inoculation on milks

The amount of 1% protease was poured into 25 mL skim and whole milks, and then it was kept at refrigerated room. Protease inoculated on milks was crude protease and semi-purified protease with ethanol 70%. Milks treated was centrifuged on 8400 g, at temperature 4° C for 5 minutes, and it was measured protease activities on 0 day (4 days before use by date), at the use by date, 4 days after the use by date, and 8 days after the use by date.

RESULTS AND DISCUSSION

The results of the protease activities of the treated milks show that the longer the storage times the higher protease activities of milks. This may be because at the time of storage 12 days, there were the highest growth of *Pseudomonas fluorescens* in pasteurized milks at storage, than that at the other times of storage. As a result, there were the highest activities of the proteases of *Ps. fluorescens* at storage 12 days than that at the other times of storage. It has been reported that the longer the times of storage, the higher the growth of *Psrudomonas fluorescens* (Chandler *et al.*, 1990; Craven and Macauley, 1992), and the longer the time of storage the higher the activities of protease in pasteurized milks (Deeth *et al.*, 2002; Hsu, 1984).

At storage 12 days, protease activities on control was 0.2556 Unit/mL (skim) and 0.2116 Unit/mL (whole), and on inoculated milks (crude) was 2.2044 Unit/mL (skim) and 1.5378 Unit/mL (whole); while on inoculated milks (semi-purified) was 3.5355 Unit/mL (skim) and 1.6778 Unit/mL (whole), respectively (Table 1-2). The higher protease activities of skim milks than whole milks may be because the higher content of protease in skim milks than whole milks. It has been reported than the lipid content of whole milks was higher than that of skim milks (Chandler *et al.*, 1990). Furthermore, the higher protease activities of skim and whole milks inoculated by semi-purified protease than that by crude protease may be because the protease activities of semi-purified protease was higher than that of crude protease. It has been reported that the activities of protease with purification was higher than that with crude (Hsu, 1984; Sorhaug and Stepaniak, 1997)

For the protein degradation on the treated milks, the results show that the longer the storage times the higher protein degradation of milks. This may be because at the time of storage 12 days, there were the highest growth of *Pseudomonas fluorescens* in pasteurized milks at storage, than that at the other times of storage. As a result, there were the highest activities of the protease of *Ps. fluorescens* at storage 12 days than that at the other times of storage. It has been reported that the longer the times of storage, the higher the growth of *Psrudomonas fluorescens* (Chandler *et al.*,1990; Allen *et al.*, 1989), and the longer the time of

Table 1. The protease activities of whole milks inoculated with protease at times of storage (Unit/mL)

No.	Times of storage	Whole milks	Inoculated	With protease
		Control (un-inoculated)	Inoculated with crude	Inoculated with semi-purified
1	0 day (4 days before use by date)	0.1058 a	0.2400 b	0.2600 c
2	4 days (at the use by date)	0.1468 d	0.6000 e	1.3214 f
3	8 days (4 days after use by date)	0.1686 g	0.7725 h	1.5025 i
4	12 days (8 days after use by date)	0.2116 j	1.5378 k	1.6778 l

Note: Different letter show significantly different (P<0.05)

Table 2. The protease activities of skim milks inoculated with protease at times of storage (Unit/mL)

No.	Times of storage	Skim milks	Inoculated	With protease
		Control (un-inoculated)	Inoculated with crude	Inoculated with semi-purified
1	0 day (4 days before use by date)	0.1263 a	0.3372 b	0.4481 c
2	4 days (at the use by date)	0.2351 d	1.6671 e	1.8123 f
3	8 days (4 days after use by date)	0.2442 g	1.9034 h	3.1002 i
4	12 days (8 days after use by date)	0.2556 j	2.2044 k	3.5355 l

Note: Different letter show significantly different (P<0.05)

Table 3. The protein degradation of whole milks inoculated with protease at times of storage (%)

No.	Times of storage	Whole milks	Inoculated	With protease
		Control (un-inoculated)	Inoculated with crude	Inoculated with semipurified
1	0 day (4 days before use by date)	3.26 a	3.32 b	3.43 c
2	4 days (at the use by date)	3.58 d	3.72 e	3.90 f
3	8 days (4 days after use by date)	3.67 g	4.97 h	5.26 i
4	12 days (8 days after use by date)	3.81 j	7.28 k	6.04 l

Note: Different letter show significantly different ($P<0.05$)

Table 4. The protein degradation of skim milks inoculated with protease at times of storage (%)

No.	Times of storage	Skim milks	Inoculated	With protease
		Control (un-inoculated)	Inoculated with crude	Inoculated with semipurified
1	0 day (4 days before use by date)	3.37 a	3.48 b	3.59 c
2	4 days (at the use by date)	3.69 d	3.82 e	4.21 f
3	8 days (4 days after use by date)	3.93 g	5.61 h	5.77 i
4	12 days (8 days after use by date)	4.48 j	7.28 k	7.62 l

Note: Different letter show significantly different ($P<0.05$)

Table 5. Homogeneous of whole milks inoculated with protease at times of storage

No.	Times of storage	Whole milks	Inoculated	With protease
		Control (un-inoculated)	Inoculated with crude	Inoculated with semipurified
1	0 day (4 days before use by date)	homogeneous	homogeneous	homogeneous
2	4 days (at the use by date)	homogeneous	homogeneous	homogeneous
3	8 days (4 days after use by date)	homogeneous	not homogeneous (+)	not homogeneous (++)
4	12 days (8 days after use by date)	homogeneous (+)	not homogeneous (++)	not homogeneous (+++)

storage the higher the activities of protease in pasteurized milks (Deeth *et al*.; 2002; Janzen *et al.*, 1982).

Protein degradation on control, inoculated skim milks (crude and semi-purified) on 12 days were 4.48%, 7.28% and 7.62%, while that on whole milks were 3.81%, 7.28% and 6.04%, respectively (Table 3-4). The higher protease activities of skim milks than whole milks may be because the higher content of protein in skim milks than whole milks. It has been reported than the protein content of skim milks was higher than that of whole milks (Chandler *et al.*, 1990). Furthermore, the higher protease activities of skim and whole milks inoculated by semi-purified lipase than that by crude proteases may be because the protease activities of semi-purified lipase was higher than that of crude protease. It has been reported that the activities of protease with purification was higher than that with crude (Hsu, 1984; Sorhaug and Stepaniak, 1997).

For the homogeneous of the treated milks, the results show that the longer the time of storage, the faster the decrease of milks' homogeneous. Furthermore, the decrease of homogeneous on milks inoculated with crude and semi-purified proteasee were faster than that of control, and homogeneous of whole milks inoculated with crude and semi-purified protease was better than that of skim milk, respectively (Table 5-6). This may be because the longer the time of storage, the higher the growth of *Ps. fluorescens*, and the increase of the growth of *Ps. fluorescens* may resulted in the decrease of milks' homogeneous. Furthermore, the add of both semi-purified and crude protease in skim and whole milks may resulted in the increase of the protease activities in the degradatipn of protein in skim and whole milks. Furthermore, the add of both semi-purified and crude protease in skim and whole milks may resulted in the increase of the protease activities in the degradatipn of protein in skim and whole milks. So, the increase of the degradation of the protein in skim and whole milks may resulted in the faster in the decrease of milks' homogeneous. It has been reported that the higher the protease activities, the higher the degradation of protein contents in skim and whole milks at storage (Hsue, 1984; Sorhaug and Stepaniak, 1997), The more homogeneous in whole milks inoculated with semi-purified and crude proteases than that in skim

Table 6. Homogeneous of skim milks inoculated with protease at times of storage

No.	Times of storage	Skim milks	Inoculated	With protease
		Control (un-inoculated)	Inoculated with crude	Inoculated with semipurified
1	0 day (4 days before use by date)	homogeneous	homogeneous	homogeneous
2	4 days (at the use by date)	homogeneous	not homogeneous (+)	not homogeneous (++)
3	8 days (4 days after use by date)	homogeneous	not homogeneous (++)	not homogeneous (+++)
4	12 days (8 days after use by date)	homogeneous(+)	not homogeneous (+++)	not homogeneous (++++)

milks may be because the more lipid contents in whole milks than that in skim milks. It has been reported that the lipid contents in whole milks were higher than that in skim milks (Chandler *et al.*, 1990).

CONCLUSION

The longer the storage times the higher protease activities and protein degradation of milks. At storage 12 days, protease activities on control in skim milks was higher than that in whole milks, and on inoculated skim milks (crude and semi-purified) was higher than that on whole milks. Furthermore, the decrease of inoculated milks' homogeneous were faster than that of control. Moreover, protein degradation on control and inoculated skim milks (semi-purified) at storage for 12 days were higher than that on whole milks, respectively, while that on inoculated skim milks (crude) were the same as on whole milks. It can be concluded that protein skim milks spoiled faster than whole milks, based on protease, protein degradation and homogeneous.

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