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MILK CURD PROPERTIES ATTRIBUTED TO THE APPLICATION OF A PINEAPPLE JUICE AS A COAGULANT IN CHEESE PRODUCTION

Irvan Setiadi Kartawiria[1], Brenna Elvina[2], Maria Dewi Puspitasari Tirtaningtyas Gunawan-Puteri[2]

[1] *Chemical Engineering Department, Swiss German University Tangerang 15143, Indonesia*
[2] *Food Technology Department, Swiss German University Tangerang 15143, Indonesia*
E-mail: irvan.kartawiria@sgu.ac.id

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ABSTRACT

The pineapple fruit (Ananas comosus L. Mer) has been used to develop traditional cheese products in Indonesia due to its bromelain content. The latter is a proteolytic enzyme, which is able to clot the milk leading to milk curd formation. The cheese product obtained has a major problem in terms of its bitterness. This research is focused on studying the source of bitterness in the milk curd and the effect of the different amounts of the pineapple juice addition on the milk curd characteristics. The analysis of the amino acid content shows that the milk curd made with a pineapple juice as a coagulant contains a high amount of tryptophan, proline, and other bitter amino acids. The percentage ratio of the bitter amino acid to the non-bitter one in a milk curd made with 15 ml and 40 ml of a pineapple juice as a coagulant is 50.67 % ± 0.02 % and 49.33 % ± 0.02 %, correspondingly. The increase of the pineapple juice addition (15 ml to 40 ml) to the milk is found to affect the amino acid content of the milk curd, which increases from 36546.91 ppm to 38219.580 ppm. The total amino acid content of the milk curd is found to be twice higher than that of the commercial Cheddar cheese.

Keywords: milk clot, curd, pineapple, bromelain.

INTRODUCTION

The conventional cheese production is mainly consisting of acidification, coagulation, and dehydration, followed by shaping and pressing the curd. Lactic acid bacteria or direct lactic acid is commonly used for acidification, while rennet as a proteolytic enzyme is used for coagulation [1]. A plant derived proteolytic enzyme may be used as a coagulant in several countries in Asia. *Dangke*, a type of a traditional cottage cheese-like product from Enrekang, Indonesia, is produced by heating cow's or buffalo's milk followed by addition of a papaya sap extract, containing papain and a plant derived protease as a coagulant component [2].

The other traditional Indonesian soft cheese product, *Dadiah* , is transformed by Philip [3] to hard cheese by using a pineapple juice as a coagulant. The main proteolytic enzyme which acts as a coagulant in the pineapple juice refers to bromelain, although the juice contains also peroxidase, phosphatase, protease inhibitors, and organically bound calcium [4].

The optimum temperature for bromelain amounts to 10˚C - 25˚C in an acidic environment (pH 4.6 - 5.2), it refers to 40˚C - 60˚C in a neutral one (pH 6.8 - 7.1), while under basic conditions (pH 7.5 - 9.5) it is in the range of 25˚C - 40˚C. However, 80 % of bromelain remain active at temperature of 70˚C and pH 3.5 - 3.8 compared to the enzyme activity measured at 40˚C and pH 6.8 - 7.1 [5].

A previous research shows a variety of the milk coagulating properties of the pineapple crude extract. The introduction of a crude juice volume of 10 ml, 25 ml, and 40 ml to 1 l of milk in the course of 30 min at a temperature of 65˚C increases the curd yield, but shows no effect on the firmness of the curd [6]. The addition of 45 ml of a pineapple extract to 1 l of milk results in cheese of the highest protein content and best flavour acceptance

[7]. Besides its curding properties, the pineapple extract may contribute to the favourable aroma of the cheese. It is composed of up to 300 different volatile compounds, which can be detected by GC/MS, although only some of them have been reported as key contributors of the pineapple juice aroma. Methyl 2-methyl butanoate and methyl hexanoate [8, 9] are the components determining the pineapple juice aroma. The thermal treatment of the pineapple juice affects the volatile compounds composition which are synthesized from free amino acids and carbohydrates as well as by β-oxidation of fatty acids [10].

In general, cheese products prepared with papain and bromelain as coagulants have a low acceptance mainly because of their flavour. The bitter after taste is well detected during the sensory evaluation [3]. The common approach to solving the biter taste problem is connected with the decrease of the amount of the enzyme used, as the latter usually has a bitter taste. The milk curd formation refers to the gradual breakdown of the milk protein into smaller sized peptides and amino acids leading to cheese of a softer texture and enhanced flavour development [1]. The proteolysis is the most essential biochemical process for flavour and texture formation in hard-type and semi-hard-type cheeses. The residual coagulant enzyme left after the curding process may survive throughout the scalding process and hence affect the cheese flavour in the ripening stage. Bromelain has a proteolytic activity which provides both hydrophilic and hydrophobic amino acids [11]. It is recognized that the bitterness is attributed to the hydrophobicity of the amino acid content as more hydrophobic peptides provide a more intense bitter flavour [12]. Furthermore, the presence of base amino acids in the amino end, the presence of proline and bigger sized peptides are likely to cause also bitterness [13].

This research is focused on studying the source of bitterness in the milk curd upon addition of bromelain in the form of a pineapple juice and the effect of varying the juice addition on the milk curding properties. The amino acid amount based on its hydrophobicity connected with the bitterness observed is quantified. This information is valuable for the development of a more systematic approach to cheese production using a plant derived proteolytic enzyme, especially from pineapple.

EXPERIMENTAL

Pineapple of Honi variety (Sunpride, Indonesia) was used in this research. It was obtained from the local market in Tangerang, Indonesia. The fruits were peeled, sliced, and then macerated using a household blender. Muslin cloth was used for the filtering. The juice obtained was chilled to 5°C prior of use. Its enzymatic activity was measured by the method of Cupp-Enyard [14] modified by Philip [3]. A solution of 250 μl of casein 1 % (Sigma-Aldrich, USA) and 250 μl of a phosphate buffer of a pH value of 7 were mixed in a microtube. Then 125 μl of 50 % crude pineapple juice solution and 125 μl of 0.04 mM $CaCl_2$ solution were added to the microtube. This step was followed by incubation for 20 min at 37˚C. It was stopped by adding 750 μl of TCA solution. The mixture was centrifuged at 8000 rpm in the course of 10 min at 4˚C (Rotina 35 R, Germany) and the resulting supernatant was transferred to a test tube. The liquid was then mixed with 1000 μl of 0.5 M Na_2CO_3 and 200 μl of Folin Ciocalteau (1:2) reagent (Sigma-Aldrich, USA). The sample was then transferred to a cuvette and the absorbance was measured at 578 nm by using a spectrophotometer (Genesys 10UV Thermo Electron, USA). The absorbance recorded was converted to activity based on the tyrosine standard curve.

The milk-clotting activity was determined by the procedure described by Arima et al. [15]. A substrate consisting of 10% skimmed milk (Prolac, French) in 0.01 M $CaCl_2$ was prepared. Then, 2 ml of the substrate were preheated in a test tube for 5 min at 37°C in a water bath, and an enzyme extract of a volume of 0.2 ml was added. The curd formation proceeded at 37°C in a water bath. The test tube was slowly manually rotated from time to time. The end point of the milk clotting was recorded by the clot appearance. The milk-clotting unit was defined as the amount of the enzyme that provided clotting of 2 ml of the substrate within 40 min.

The milk used for its curd properties investigation was obtained from a farmer in Jakarta, Indonesia. The fresh milk of a volume of 500 ml was heated in a water bath until it reached 40˚C. Pineapple juice portions of 15 ml and 40 ml were added while the temperature was maintained at 40˚C. The addition of the coagulant was accompanied by stirring. Thus an even distribution of the coagulant was achieved. The milk mixture was then

Table 1. Unit of the enzyme activity of a pineapple juice solution obtained from three different fruits.

Sample	Tyrosine released (μmol)	Enzyme activity (U/ml)
A	1.556	1.037
B	1.536	1.024
C	1.513	1.009
Average		1.023 ± 0.011

put to rest for clotting and curdling. The amino acid was determined using UPLC (ACC Q-Tag Ultra C18 column at a temperature of 49°C, with PDA detector at 260 nm wavelength), by Saraswanti Indo Genetech Lab, Indonesia.

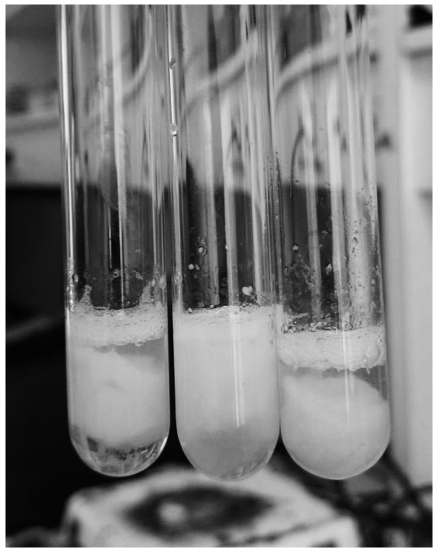

Fig. 1. A milk clotting activity of a pineapple juice obtained from three different pineapple fruits.

RESULTS AND DISCUSSION

Enzyme activity measurement

The unit of enzyme activity (U) has to be determined in order to eliminate the inconsistency caused by the different quality of the fruit used. U is defined as the peptide amount released per minute (mol/ min). Although the pineapple used in this research is obtained from an identical source, its proteolytic enzyme activity may differ due to the different storage, the season, or the post-harvest handling. Table 1 shows the results of the enzymatic activity of three different fruits where only about 1.07 % differences are observed among the samples. This result shows that the pineapple enzymatic property is relatively consistent and there is a low variation among the fruits in term of the bromelain content.

The amount of the pineapple juice used for clotting evaluation is 15 ml and 40 ml (15.349 U and 40.930 U, respectively). It is determined on the ground of a previous research [6, 7]. The conversion of the volumetric measure to an enzyme activity unit is important because of the common practice to use the volumetric ratio of a pineapple juice to milk. This finding is providing a measure of standardization in respect to the previous study, although a different variety of a pineapple has been used.

Milk clotting and protease activity

The milk clotting activity is determined using two different milk curds coagulated after a pineapple juice addition. As illustrated in Fig. 1, the observation is done when the milk clots with a slight yellow liquid appearance indicating the separation of the insoluble from the soluble protein particles. In this case 0.2 ml of a pineapple juice are added to 2 ml of a substrate at 37˚C. Casein is the insoluble protein which forms the milk curd, while whey is the soluble protein alongside with other soluble components of the milk in the yellow liquid.

The results referring to the milk clotting activity of three samples of a pineapple juice are shown in Table 2.

Table 2 shows that the milk clotting activity obtained with three different pineapple fruits amounts to 115.976 ± 13.643 U/ml.

The ratio of the enzymatic activity and the milk clotting activity can be used to predict the characteristics of the curd produced. An enzyme with a higher ratio of a milk-clotting to a proteolytic activity is more suitable to form a curd of a higher yield and less bitterness during cheese processing, while that with low ratio may develop a lower curd recovery and a weak curd firmness. It can also release bitter peptides that affect the gustatory properties of the final product [16]. The comparison between bromelain and other milk clotting enzymes [17] is illustrated in Table 3. It is evident that bromelain has the lowest ratio. The latter is one of the reasons that most of Indonesian traditional cheese products (e.g. Dangke and Dadiah) are in the form of soft cheese of an observable bitterness. The low ratio indicates also that the cheese product bitterness can be

Table 2. A milk clotting activity of three different pineapple fruits.

Sample	Milk Clotting Time (s)	Milk Clotting Activity (U/ml)
A	233	103.004
B	178	134.831
C	218	110.092
Average	209.667	115.976 ± 13.643

attributed to the peptides content, but not merely to the taste of the juice itself.

Amino acid profile

The amino acid profile is determined to evaluate the amount of non-bitter and bitter peptides aiming to confirm the hypothesis that peptides content determines the bitterness of the milk curd. Table 4 shows the analytical results referring to the non-bitter peptides.

Table 4 shows that the total non-bitter amino acids content increases from 18036.03 ppm to 18847.33 ppm with increase of the pineapple juice addition. The statistical analysis (t-test) referring to the milk curd made with 15 ml and 40 ml of a pineapple juice is not significantly different (p-val = 0.07107 ≥ 0.05).

The cysteine amount decreases from 164.23 ppm to less than 161.24 ppm with the pineapple juice addition increase from 15 ml to 40 ml. This phenomenon is suggested to be attributed to pH decrease as the pineapple juice pH is 3.71 [8]. Thus the amino acids are removed from their isoelectric point, which results in their solubility increase. That is why they leave the milk curd alongside with the whey solution.

The bitter peptides analysis is illustrated in Table 5. A high increase of several peptides is observed, although the total increase of the bitter amino acid content in the milk curd made with introduction of 15 ml and 40 ml of a pineapple juice shows no significant difference (p = 0.11869 ≥ 0.05).

7 bitter amino acids with the exception of lysine and tryptophan are present in a higher amount (ppm) in sample 2 rather than in sample 1. The decrease of lysine and tryptophan amounts in the milk curd made with the introduction of 40 ml of a pineapple juice is due to oxidase and hence will decrease the protein quality of casein. The effect on the Maillard the excessive enzymatic reaction of polyphenol oxidase, which exists naturally in the pineapple fruit. The increased pineapple juice addition results in a higher amount of polyphenol reaction will be similar. Lysine and tryptophan are reported to be drastically decreased because of the effect of polyphenol oxidase. The value (50 %) observed is greater than that of the other amino acids, such as histidine and tyrosine (14 % - 22 %) [18]. The total amino acid content increases from 36546.91 ppm to 38219.58 ppm with increase of the pineapple juice addition from 15 ml to 40 ml, respectively. The amino acid content of the milk curd in sample 1 (15 ml) and sample 2 (40 ml) are proven to be significantly different in view of the the paired t-test. The difference is determined by the p-value of 0.01388 (< 0.05). Therefore, the increase of

Table 3. A ratio of the milk clotting activity and the proteolytic activity observed in case of several enzymes and a pineapple juice used as coagulants.

Enzyme	Milk Clotting Activity (Unit/ml)	Protease Activity (unit/ml)	Ratio
Rennet	249.6	0.05	4992
Dubiumin	880	0.35	2490
Papain	216	0.59	367
M. oleifera seed extract	13407.8	2.5	5279
Pineapple juice	*115.976*	*1.023*	*113.369*

Table 4**.** A non-bitter amino acid content of a milk curd obtained with the addition of 15 ml and 40 ml of a pineapple juice as a coagulant.

Non-Bitter Amino Acids	A pineapple juice addition of	
	15 ml	40 ml
	(ppm)	(ppm)
Alanine	1272.55	1288.77
Arginine	1214.9	1334.29
Aspartic acid	2530.42	2625.08
Cysteine	164.23	<161.24
Glutamic acid	7700.07	8030.05
Glycine	764.63	803.49
Histidine	960.29	1089.13
Serine	1977.41	2111.62
Threonine	1451.53	1564.9
Total amino acid content	18036.03	18847.33

Table 5. A bitter amino acid content of a milk curd made with the addition of 15 ml and 40 ml of a pineapple juice addition as a coagulant.

Bitter Amino Acids	A pineapple juice addition of	
	15 ml (ppm)	40 ml (ppm)
Proline	3116.19	3321.84
Tyrosine	1830.94	2039.4
Leucine	3315.61	3496.23
Lysine	3475.81	3363.81
Valine	1954.18	2098.2
Isoleucine	1595.83	1725.15
Phenylalanine	1895.37	2178.08
Methionine	753.19	795.59
Tryptophan	573.76	353.95
Total content	18510.88	19372.25

the amino acid content of the milk curd depends on the amount of the pineapple juice introduction to the milk. However, the contents of the bitter and the non-bitter amino acid produced by using any levels of a pineapple juice as a coagulant refer to 50.67 % ± 0.02 % and 49.33 % ± 0.02 %, correspondingly. Table 6 shows that the peptides profile of the commercial cheddar cheese [19] made with rennet as a coagulant shows a higher percentage of bitter amino acids presence (52.11 %) than that of non-bitter one (47.89 %). The individual profile may be however explained with the pineapple juice as a source of milk curd bitterness.

The total amino acid content, both of non-bitter and bitter amino acids, is almost doubled in the product obtained with a pineapple juice as coagulant. The non-bitter amino acid content of the milk curd is shown to be richer as histidine is detected, while it is absent in Cheddar cheese. The glutamic acid, amino acid that contributes to the desirable flavour is also found significantly increased in product made with a pineapple juice. This profile shows the potential of the milk curd considered in respect to a richer flavour. Furthermore, the presence of non-bitter amino acid can be increased upon subsequent separation and purification. On the other hand, based on the classification of the non-bitter and bitter peptides according to the Q values (an average hydrophobicity of a peptide), the amino acids of a Q value lower than 1300 cal res^{-1} are not bitter, while amino acids of a Q value higher than 1400 cal res^{-1} provide a bitter flavour. Tryptophan and proline are considered the most bitter amino acids [13]. The amount of tryptophan and proline as the most bitter amino acids in the milk curd made with a pineapple juice is very high (573.76 ppm and 3116.190 ppm, respectively, in case of 15 ml of a juice addition). It is worth adding that tryptophan is not detected (0 ppm), while proline is only 286.445 ppm in Cheddar cheese.

This is likely to be the biggest problem in respect to the bitterness of the milk curd made with a using pineapple juice as coagulant.

Table 6. An amino acid composition of Cheddar cheese and a milk curd made with 15 ml and 40 ml of a pineapple juice as a coagulant.

Amino Acid	Cheddar cheese (ppm)	A pineapple juice addition of 15 ml (ppm)	A pineapple juice addition of 40 ml (ppm)
Bitter amino acid			
Isoleucine	404.898	1595.830	1725.150
Leucine	2396.590	3315.610	3496.230
Lysine	1006.980	3475.810	3363.810
Methionine	386.745	753.190	795.590
Phenylalanine	1326.840	1895.370	2178.080
Proline	286.445	3116.190	3321.840
Tryptophan	0.000	573.760	353.950
Tyrosine	611.625	1830.940	2039.400
Valine	938.477	1954.180	2098.200
Non-bitter amino acid			
Alanine	286.840	1272.550	1288.770
Arginine	1001.360	1214.900	1334.290
Aspartic acid	1328.100	2530.420	2625.080
Cysteine	38.517	164.230	<161.240
Glutamic acid	2769.520	7700.070	8030.050
Glycine	235.980	764.630	803.490
Histidine	0.000	960.290	1089.130
Serine	348.000	1977.410	2111.620
Threonine	382.158	1451.530	1564.900
Total amino acid	13749.075	36546.910	38219.580

CONCLUSIONS

A pineapple juice may be used as a coagulant in the milk curding process although the ratio of the proteolytic activity and the milk clotting is low (113.369) in comparison to that in presence of other proteolytic enzymes. The evaluation of the amino acid profile shows that the source of the bitterness refers not only to the ratio of bitter and non-bitter peptides, but predominantly to the bitter amino acids concentration. Tryptophan and proline known for their greatest contribution to the bitter flavour are found in high amounts (573.76 ppm and 3116.190 ppm, respectively, in case of 15 ml of a juice addition). The total amino acid content of the milk curd made with a pineapple juice is higher than that in presence of rennet. This fact indicates that the fruit juice studied can be explored as a specific source of amino acids.

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