Studies on nucleic acid derivatives in cheese

II. The changes of acid-soluble nucleic acid derivatives in Blue, Gouda and Cheddar cheeses during ripening

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Cheeses are ripened by rennin and other various enzymes which exist naturally in milk or originate from microorganisms, and their characteristic flavors are produced. It is also considered that the intracellular nucleotides of microorganisms in cheeses are exuded out from cells by autolysis and play partially a role in the specific flavor-production of cheeses.

In the previous paper⁷⁾, the nucleotides found in the white mold cheese after 21 days' ripening were 3'-CMP (3'-cytidine monophosphate), cyclic CMP, 5'-UMP (5'-uridine monophosphate), GTP (guanosine triphosphate), UTP (uridine triphosphate), ATP (adenosine triphosphate) and CTP (cytidine triphosphate) and their contents were 8.6, 13.8, 4.7, 3.2, 4.7, trace and 25.9 μM /100 g, respectively. It was also found that the total content of nucleotides had a close relationship to the stage of cheese-ripening and was presumed that the appearance of these nucleotides in cheeses was originated from the autolysis of microorganisms.

It is known that the starter used and the ripening conditions of cheese are not the same in each cheese, so the pattern of changes of acid-soluble nucleotides may presumably differ in each cheese. In the present work, the authors have carried out a study on the changes of acid-soluble nucleic acid derivatives of Blue, Gouda and Cheddar cheeses during ripening.

Methods and Materials

1. The cheese samples

Blue, Gouda and Cheddar cheeses prepared by the conventional methods were supplied by Snow-Brand Milk Products Co., Ltd. Gouda and Cheddar cheeses were ripened at 15°C, and Blue cheese at 10°C under 80-90 % relative humidity in the ripening room. During the ripening period, the experimental samples were taken periodically and ground in a mortar.

2. Chromatography of acid-soluble nucleic acid derivatives

Twenty grams of ground cheese was extracted by blending with 50 ml chilled 10% HClO_4 for 5 minutes in a laboratory homogenizer (Nihon Seiki Kogyo, HD-type), and centrifuged for 10 minutes at 2500 r.p.m. The residue was reextracted in the same manner with 50 ml of HClO_4 . These extracts, which contained acid-soluble nucleic acid derivatives, were combined and immediately neutralized with 5 N KOH to pH 6.5 in an ice-bath. After being kept at 0°C for about 30 minutes to permit the complete precipitation of KClO_4 , the neutralized extract was centrifuged to remove KClO_4 and made to 250 ml with distilled water.

A portion of the neutralized extract was added to the column (Dowex 1-X 8, formate, 200-400 mesh, 1×10 cm) and then the column was washed with 200 ml of distilled water before starting the elution. The method of the elution of nucleotides from the column, as shown in

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Fig. 1, was similar to that given in the previous report⁷⁾.

Each fraction was absorbed by activated charcoal, washed with 50 ml of 2 N formic acid and then with water until the washing became neutral. It was finally extracted with 50 ml of 50% ethanol containing 2% ammonia. The extract was concentrated and desalted with Dowex 50-X 12 (H-form). The desalted extract was evaporated to dryness in vacuum and used for paperchromatography.

The solvent systems used were as follows:

- A. Iso-propanol: Saturated ammonium sulfate: 0.1 M acetate buffer of pH 6.0 (2 : 79 : 19 by volume)
- B. Methanol: HCl: Ethanol: Water (50 : 6 : 25 : 19 by volume)
- C. Iso-butyric acid: 1 M ammonia: 0.1 M EDTA (100 : 60 : 1.6 by volume)
- D. Iso-butyric acid: 0.5 M ammonia (10 : 6 by volume, pH 3.6)
- E. Iso-butyric acid: Water: Acetic acid (100 : 50 : 1 by volume)

The solvent systems A, B and C were used for nucleotide monophosphate, C, D and E for nucleotide polyphosphate. The nucleotides were detected on paperchromatograms by using UV light (253.6 m μ).

For the identification of nucleotides, authentic substances were subjected to column and paper chromatography together with the extracts from cheese samples. The determination of the molar ratio of phosphorus to ribose, and the identification of the bases of nucleotides were also done with chromatographically separated fractions. Phosphorus and ribose contents of nucleotides were determined by molybdate reagent method of WELL-MALHERBE and GREEN¹²⁾ and the orcinol reagent method of ALBAUN and UMBREIT¹¹⁾, respectively. For the identification of the bases of nucleotides, the above mentioned concentrated sample was hydrolyzed with 1 N HCl for 1 hour at 100°C and submitted to paper chromatography with solvent systems A and B.

The quantities of nucleotides were estimated by using the following molar absorbcancy indices at pH 2.0¹¹⁾:

Adenosine phosphates 14.2×10^3 ; Guanosine phosphates 11.8×10^3 ; Inosine phosphates 7.4×10^3 ; Cytosine phosphates 6.2×10^3

3. Determination of phosphatase activity of cheeses

For the determination of phosphatase activity, the method of BESSEY *et al.*³⁾ was used with a slight modification. One gram of ground cheese was extracted with 50 ml of distilled water by using a homogenizer, and filtered. The filtrate was made to 100 ml with distilled water and used as an enzyme solution.

Two ml of buffer solution (pH 10.5, glycine-NaOH), 2 ml of 0.04% substrate solution (p-nitrophenyl phosphate) and 2 ml of enzyme solution were mixed at 38°C and incubated for 60 minutes. Then, 4 ml of 0.5 N NaOH was added and the developed color intensity was measured at 420 m μ .

One μ M of p-nitrophenol liberated per gram cheese per hour at 38°C was defined as one unit of phosphatase activity.

4. Analysis of cheese

Moisture, titratable acidity, pH, total nitrogen, water soluble nitrogen, ammonia nitrogen and tyrosine were determined by the conventional methods⁸⁾.

Results and Discussion

The changes of chemical composition of Blue, Gouda and Cheddar cheeses during ripening

were shown in Tables 1, 2 and 3.

From these results the ripening of the cheeses is considered to have proceeded normally. The most suitable ripening periods were 60 days for Blue cheese and 120 days for Gouda and Cheddar cheeses, respectively, when judged from flavor and taste of cheeses.

Table 1. The changes of chemical composition of Blue cheese during ripening

Period of ripening (days)	0	10	20	30	40	60	90
Moisture (%)	42.74	42.16	43.72	43.56	43.52	42.45	41.51
Acidity* (%)	2.61	2.57	1.71	1.53	1.44	1.35	1.35
Protein (%)	20.55	21.76	22.20	21.44	21.63	21.76	22.65
pH	4.9	4.9	5.7	6.5	6.9	7.2	7.5
Total nitrogen (%)	3.22	3.41	3.48	3.36	3.39	3.41	3.55
Water soluble nitrogen (%)	0.32	0.37	0.90	1.31	1.58	1.73	2.13
Ammonia nitrogen (%)	0.04	0.06	0.07	0.15	0.21	0.24	0.43
Tyrosine (mg%)	60.0	68.0	135.0	205.0	225.0	230.0	425.0
Ripening ratio** (%)	10.04	10.85	25.86	38.99	46.61	50.73	60.00

* Calculated as lactic acid

** Ripening ratio: water soluble nitrogen/total nitrogen $\times 100$

Table 2. The changes of chemical composition of Gouda cheese during ripening

Period of ripening (days)	0	30	60	90	120	150	180
Moisture (%)	38.72	36.96	36.60	34.55	35.82	36.75	36.82
Acidity (%)	0.72	1.35	2.12	1.80	1.89	1.80	2.16
Protein (%)	27.37	28.26	28.84	28.90	27.94	28.52	28.14
pH	5.9	5.9	5.8	5.9	5.9	5.9	5.8
Total nitrogen (%)	4.29	4.43	4.52	4.53	4.38	4.47	4.41
Water soluble nitrogen (%)	0.38	0.75	1.22	1.25	1.23	1.34	1.35
Ammonia nitrogen (%)	0.05	0.06	0.08	0.10	0.10	0.10	0.09
Tyrosine (mg%)	36.0	72.0	96.0	120.0	145.0	155.0	160.0
Ripening ratio (%)	8.81	16.93	26.99	27.59	28.08	29.98	30.61

Table 3. The changes of chemical composition of Cheddar cheese during ripening

Period of ripening (days)	0	30	60	90	120	150	180
Moisture (%)	36.07	37.08	36.27	36.20	36.34	37.01	36.12
Acidity (%)	1.08	2.79	2.61	2.16	2.16	2.07	2.16
Protein (%)	27.16	27.31	27.82	27.94	27.12	27.37	27.70
pH	5.5	5.4	5.4	5.6	5.5	5.6	5.5
Total nitrogen (%)	4.25	4.28	4.36	4.38	4.25	4.29	4.34
Water soluble nitrogen (%)	0.79	0.89	0.97	1.03	1.01	1.07	1.22
Ammonia nitrogen (%)	0.05	0.06	0.08	0.09	0.10	0.10	0.07
Tyrosine (mg%)	51.0	77.0	108.0	132.0	155.0	165.0	205.0
Ripening ratio (%)	18.59	20.79	22.31	23.52	23.75	24.94	28.11

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Fig. 1. Elution pattern of acid-soluble nucleic acid derivatives of Blue cheese (20 g) ripened for 60 days on the column chromatography

The column chromatography was carried out using a column (1.0×10 cm) containing an anion exchange resin Dowex 1-X 8 (200-400 mesh) formate form. Flow rate was 1.5 ml per minute. Each 10 ml fraction was collected in a tube.

Elution solutions were as follows: A, 0.005 M formic acid (FA). B, 0.02 M FA. C, 0.1 M FA. D, 0.1 M FA+0.1 sodium formate (FNa). E, 0.1 M FA+0.6 M FNa. F, 0.1 M FA+0.8 M FNa. G, 0.5 M FA+1.0 M FNa. H, 2.0 M FA+1.0 M FNa

I, Inosine; II, 5'-CMP; III, 5'-AMP; IV, 5'-IMP; V, GTP; VI, ADP; VII, ATP; VIII, Guanosine

Fig. 2. Elution pattern of acid-soluble nucleic acid derivatives of Gouda cheese (20 g) ripened for 120 days on the column chromatography

The conditions of column chromatography were the same as given in Fig. 1.

V, GTP; VI, ADP; VII, ATP; VIII, Guanosine

Fig. 3. Elution pattern of acid-soluble nucleic acid derivatives of Cheddar cheese (20 g) ripened for 120 days on the column chromatography

The conditions of column chromatography were the same as given in Fig. 1.

I, Inosine; V, GTP; VII, ATP; VIII, Guanosine

Fig. 1, 2 and 3 represent the elution patterns of acid-soluble nucleic acid derivatives of Blue (ripened for 60 days), Gouda and Cheddar (ripened for 120 days) cheeses, respectively. In these patterns, the acid-soluble nucleic acid derivatives identified by paper chromatography

and the estimation of the molar ratio among base, ribose and phosphorus were as follows: I. inosine, II. 5'-CMP, III. 5'-AMP (5'-adenosine monophosphate), IV. 5'-IMP (5'-inosine monophosphate), V. GTP, VI. ADP (adenosine diphosphate), VII. ATP, VIII. guanosine. The identified acid-soluble nucleic acid derivatives in cheeses were nucleotides except two nucleotides of inosine and guanosine. In this report, for convenience, the ASND stands for the acid-soluble nucleic acid derivatives.

The changes of ASND of Blue cheese during 90 days' ripening were shown in Table 4. In the early stage of ripening, seven ASND were detected, five of which were adenine derivatives, namely, ATP, ADP, 5'-AMP, 5'-IMP and inosine. The content of 5'-IMP increased from 1.9 μM at the beginning day of ripening to its maximum value of 3.1 μM after 30 days' ripening, then decreased to 1.4 μM (0.5 mg % in cheese) after 40 to 60 days' ripening, and again slightly increased after 90 days' ripening. The total content of ASND was the largest after 10 days' ripening (41.6 $\mu\text{M}/100\text{g}$). With the changes of the total content of ASND the growth of ino-

Table 4. The changes of contents of acid-soluble nucleic acid derivatives (ASND) in Blue cheese during ripening

Fraction	ASND	Period of ripening (days)						
		0	10	20	30	40	60	90
		— $\mu\text{M}/100\text{g}$ —						
I	Inosine	3.4	4.2	0.6	0.8	0.8	0.7	0.7
II	5'-CMP				0.2	0.3	0.6	0.8
III	5'-AMP	3.7	7.6	0.3	0.7	0.6	0.4	0.7
IV	5'-IMP	1.9	2.0	1.7	3.1	1.4	1.4	2.0
V	GTP	5.8	22.1	7.9	3.4	3.3	2.7	3.6
VI	ADP	1.1	1.2	0.4	1.6	1.0	0.8	2.2
VII	ATP	3.9	3.2	5.5			0.5	1.5
VIII	Guanosine	2.6	1.3	1.7			1.2	1.3
Total		22.4	41.6	18.1	9.8	7.4	8.3	12.8

Table 5. The changes of contents of acid-soluble nucleic acid derivatives (ASND) in Gouda cheese during ripening

Fraction	ASND	Period of ripening (days)						
		0	30	60	90	120	150	180
		— $\mu\text{M}/100\text{g}$ —						
I	Inosine	4.3	3.9					
II	5'-CMP	2.8	0.5					
III	5'-AMP	1.9						
IV	5'-IMP	4.2						0.7
V	GTP	2.3	0.7	0.5	0.5	0.4	0.6	0.8
VI	ADP					0.3	0.3	0.2
VII	ATP		1.2	0.5	0.4	0.3	0.4	0.2
VIII	Guanosine			1.9	1.6	1.4	1.4	1.3
Total		15.5	6.3	2.9	2.5	2.4	2.7	3.2

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Table 6. The changes of contents of acid-soluble nucleic acid derivatives (ASND) in Cheddar cheese during ripening

Fraction	ASND	Period of ripening (days)					
		0	30	60	90	120	180
		— $\mu\text{M}/100\text{g}$ —					
I	Inosine	5.7	4.5	0.5	0.4	0.3	0.8
II	5'-CMP	0.3	0.3				0.3
III	5'-AMP						0.4
IV	5'-IMP						0.6
V	GTP	2.4	0.7	2.2	0.3	0.7	0.8
VI	ADP	0.8	0.4	0.8	0.3		0.4
VII	ATP			2.1	0.4	0.6	
VIII	Guanosine			3.7	1.1	1.0	
Total		9.2	5.9	9.3	2.5	2.6	3.3

culated *Penicillium roqueforti* seems to have been correlated. Namely, since the growth of *P. roqueforti* is known to become evident inside the cheese within 8 to 10 days' ripening and gradually propagate to reach its maximum development throughout the curd particles within 30 to 90 days' ripening³), for the mold propagation ASND derived from the autolysed cells were probably utilized rapidly at the earlier stage of ripening, after which a slight decrease in the ASND content followed, but as the growth of mold was presumably limited at the further advanced stage of ripening, the content of ASND slightly increased again after 90 days' ripening.

Table 5 represents the changes of ASND in Gouda cheese. At every ripening period, 3 to 5 ASND were detected. In the early stage of ripening, nucleotide monophosphates occupied most part of the total ASND, but as the ripening proceeded they disappeared, and replacing them nucleotide polyphosphates appeared. As an exception, 5'-IMP was found in both of the cheeses ripened for 0 and 180 days, and its content was 4.2 and 0.7 μM , respectively. The total content of ASND was the largest (15.5 μM) at the beginning day of ripening.

Table 6 shows the changes of ASND in Cheddar cheese. The ASND in cheese ripened for 60–120 days belonged mostly to nucleotide polyphosphates. In the cheese ripened for 180 days, three nucleotide monophosphates, including 5'-IMP, appeared. That the total content of ASND was the largest in the green Cheddar cheese at the beginning day of ripening was similar to Gouda cheese. In Gouda and Cheddar cheeses, ASND were supposed to be originated from the starter organisms, and in both cheeses the total content of ASND tended to decrease with the ripening period in general and to increase again slightly at the final stage of ripening in a similar manner.

During the ripenig of Blue, Gouda and Cheddar cheeses, eight kinds of ASND were found. Five of them were adenine derivatives. The following pathway of adenine derivatives have been known in the process of autolysis of tissue of fish: $\text{ATP} \rightarrow \text{ADP} \rightarrow \text{AMP} \rightarrow \text{IMP} \rightarrow \text{inosine}$ ⁵). This pathway, however, was not clearly observed in this study of cheese ripening. Probably because the autolysis and metabolism of microorganisms had proceeded simultaneously, any appreciable amount of 5'-IMP may have not accumulated.

In this study, though 5'-IMP which is an important flavoring constituent of animal tissue was found in cheeses, its amount was far below the threshold value of taste (0.0125%).

But, as synergistic action is known between 5'-IMP and monosodium L-glutamate⁶⁾, the threshold value 5'-IMP may have presumably been reduced in the presence of free glutamic acid produced by the ripening of cheese, and 5'-IMP may have exhibited some effects on the flavor of cheese.

It is known that physical, chemical and microbiological changes take place throughout the ripening period in all kinds of cheeses Fig. 4 and 5 represent the changes of the ripening ratio, tyrosine content and total content of ASND.

Fig. 4. The changes of total content of acid-soluble nucleic acid derivatives (ASND), ripening ratio and tyrosine content in Blue cheese

Fig. 5. The changes of total content of acid-soluble nucleic acid derivatives (ASND), ripening ratio and tyrosine content in Gouda and Cheddar cheeses

These results showed that the total content of ASND in each cheese varied in its respectively different manner, while the tyrosine content increased consistently in all cheeses with the ripening period. The variation of ASND content may have probably been due to the microbiological changes. In comparison with other unripened or over-ripened cheeses, the content of total ASND of moderately ripened cheeses usually stands lower. In Blue cheese, the growth of *P. roqueforti* is evident after 8 or 10 days' ripening⁹⁾. The maximum amount of total ASND

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in Blue cheese was observed at 10 days' ripening. In Gouda and Cheddar cheeses, where only lactic acid starter is used, the number of lactic acid bacteria is said to decrease during ripening⁴⁾. In Fig. 5, the content of ASND decreased gradually during ripening.

Because ASND are thought to have been derived from microbial cells, it is natural that the content of ASND in Blue cheese, in which a great number of fungi cells are present, is larger than those in Gouda and Cheddar cheeses. In Gouda and Cheddar cheeses, ASND seemed to be nothing but minor components.

As the ripening proceeded, the decrease in the content of ASND was observed. The presence of phosphatase, which decomposes nucleotides to nucleosides, is supposed to be one of the causes of the decrease in ASND. The changes of phosphatase activities in cheeses during ripening were shown in Tables 7 and 8.

Table 7. The changes of phosphatase activities in Blue cheese during ripening

Period of ripening (days)	0	10	20	30	40	60
The phosphatase activities*	0.15	0.16	0.22	0.22	0.28	0.28

* Expressed as μ M of p-nitrophenyl liberated from buffer substrate (pH 10.5) per gram of cheese sample per hour at 38°C

Table 8. The changes of phosphatase activities in Gouda and Cheddar cheeses during ripening

Period of ripening (days)	0	30	60	90	120	150	180
The phosphatase activities*							
Gouda	0	0	0	0	0	0	0
Cheddar	0.50	0.47	0.40	—	0.19	0.17	0.20

* Expressed in the same manner as described in Table 7.

The phosphatase activity was noticed in both cheeses of Blue and Cheddar, but not in Gouda cheese. During ripening, the phosphatase activity increased in Blue cheese, but decreased in Cheddar cheese. This difference seemed to correlate to the changes of the number of microbial cells during ripening, that is, in Blue cheese, *P. roqueforti* may have continued to grow, while, in Cheddar and Gouda cheeses, the number of lactic acid bacteria may have decreased⁴⁾.

In Gouda cheese, in spite of no phosphatase activity, the decrease of ASND was observed. Besides phosphatase, in this case, the utilization of ASND by microorganisms may be considered as a cause of the decrease in ASND. It has been reported by TSUGO and TANIGUCHI⁹⁾ and TANIGUCHI *et al.*¹⁰⁾ that some kinds of nucleotides are utilized as growth stimulant by lactic acid bacteria.

Many factors, such as the number of microbial cells, the decomposition by phosphatase or utilization by microorganisms, affect the changes of ASND in cheese, and the decreasing tendency of ASND with ripening period as shown in Fig. 4 and Fig. 5, indicates that the amount of ASND decomposed or utilized was larger than that derived from microbial cells. When the growth of microorganisms is very prominent, the amount of ASND derived from cells may overcome that of ASND decomposed or utilized. An example of this is the semi-soft white mold cheese, in which ASND continued to increase linearly during one month's ripening to reach such a fairly large amount of 120 μ M per 100 g cheese⁷⁾.

Summary

The acid-soluble nucleic acid derivatives appeared in Blue, Gouda and Cheddar cheeses

during ripening were fractionated by ion-exchange column chromatography. Each fraction obtained was identified by means of determining phosphorus, ribose and bases, and submitting to paper chromatography.

The acid-soluble nucleic acid derivatives detected in these cheeses throughout the ripening period were inosine, 5'-CMP, 5'-AMP, 5'-IMP, GTP, ADP, ATP and guanosine. The total content of these compounds of moderately ripened Blue, Gouda and Cheddar cheeses was 8.3, 2.4 and 2.6 μM per 100 g, respectively, and the majority of them were affiliated to adenine derivatives. The amount of 5'-IMP which is known as a flavoring component found in Blue cheese was far below the threshold value of taste. But by the synergistic action of glutamic acid presumably coexisting in cheese, 5'-IMP may have exhibited some favorable effect on cheese flavor.

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チーズ中の核酸関連物質に関する研究

II. ブルー、ゴーダおよびチェダーチーズの熟成中における酸可溶性核酸関連物質の変化

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熟成中におけるブルー、ゴーダおよびチェダーチーズの酸可溶性核酸関連物質を陰イオン交換樹脂、(Dewex 1-X8, ぎ酸型, メッシュ 200~400) を用いたカラムクロマトグラフィーにより分別し、各画分について、りん, 糖, 塩基の分析およびペーパークロマトグラフィーによって各核酸関連物質を確認定量した。この3つのチーズ中に存在する酸可溶性核酸関連物質はイノシン, 5'-CMP, 5'-AMP, 5'-IMP, GTP, ADP, ATP およびグ

アノシンの8種で、これら核酸関連物質は主としてアデニン系に属する。これらのうち、呈味成分として知られている5'-IMPの量はブルーチーズにおいてもっとも多かった。その量は5'-IMPの呈味の閾値より低いが、チーズ中では熟成の結果生成したグルタミン酸との相乗効果も考えられ、チーズ風味に影響する可能性がある。また適度の熟成状態にあるブルー、ゴーダおよびチェダーチーズの酸可溶性核酸関連物質の全量は100g当りそれぞれ8.3, 2.4および2.6 μ Mであった。

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