Isolation and Identification of Low Molecular Weight Peptides in Gouda Cheese

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Abstract 70%-Ethanol soluble extracts in Gouda cheese (cheese before ripening and cheese ripened for 1 and 4 months) were prepared to examine low molecular weight peptides in the cheese. The extracts were fractionated by high pressure liquid chromatography (HPLC) fitted with ODS column and UV detection. As the ripening period progressed, the number and quantity of peptide peaks extracted increased. The amino acid sequence of the main peaks isolated from cheese ripened for 4 months were determined as follows; Tyr, Phe, Trp, α 1-CN (f1-7), (f1-9), (f1-13), (f1-14), (f1-16), (f1-17) and β -CN (f47-52). With the exception of β -CN (f47-52) and amino acid; these peptides were fragments contained in α 1-CN (f1-23) produced specifically by the action of chymosin. Therefore, it was considered that these N-terminal peptides of α 1-CN identified in the cheese, were produced from 23 N-terminal residues of α 1-CN on the action of proteolytic enzyme from Lactic acid bacteria which existed in the cheese.

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Cheese ripening is a complex process that involves three activities: glycolysis, lipolysis, and proteolysis. Of these three, proteolysis is the most important process because it is related to the development of texture and flavor during cheese ripening^{5,6)}. Such compounds as peptides and amino acids, resulting from the proteolysis, are important constituents that affect the cheese quality. It is important to isolate and identify individual peptides generated during ripening to completely understand proteolysis in cheese. There are studies^{1,6)} on the proteolysis in cheese. How-

ever no detailed knowledge of products, especially low molecular weight (LMW) peptide, has been reported.

During the last decade, due to the good resolution of complex mixtures, reversed-phase high pressure liquid chromatography (RP-HPLC) has been widely employed in food analysis. RP-HPLC analysis can be used advantageously for cheese quality, ripening characteristics, process development such as accelerated cheese, and separation of peptides from cheese.

In this paper, LMW peptides were isolated

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and purified in Gouda cheese fractionated by HPLC, and amino acids composition were identified and N-terminal amino acids sequence was made.

Materials and Methods

Gouda cheese ripened for 1 and 4 months and unripe cheese or 0-month ripened cheese obtained from Snow Brand Milk Product Co, Ltd., were used.

Sample of Gouda cheese (20 g), which was taken below 1 cm of the cheese surface, was grated with 100 ml of distilled water and homogenized for 1 min with a Polytron PT 20 (Kinematica, Switzerland). The homogenate was then centrifuged for 30 min at 4,000 rpm to remove the cream layer. After removing the cream layer, the precipitate and supernatant were mixed, and then the cheese slurry was adjusted to pH 4.5 by the addition of 1 N HCl and stirred for 15 min. After insoluble materials were removed by centrifugation at 8,000 rpm, the pH 4.5 soluble fraction, recovered through cotton, was combined with ethanol (Et-OH) to a 70% (V/V) final concentration to eliminate the protein completely. The suspension was left to stand over 4 hr and centrifuged again at 10,000 rpm for 30 min. The clear solution was then recovered. The 70% Et-OH soluble peptides were used for HPLC analysis. All the treatments described were carried out at 4°C.

A HPLC fitted with a YMC ODS column (150 × 4.6 mm) and a UV detection at a wavelength of 220 nm was used for the separation of peptides. The 70% Et-OH soluble fraction obtained above was dried in an evaporator, dissolved in 0.1% trifluoroacetic acid and injected into a pump (LC-6 A, Shimadzu Corp., Kyoto, Japan) using an automatic injector (SCL 6 B, Shimadzu Corp., Kyoto, Japan). The injected sample was eluted with 0.1% trifluoroacetic acid-acetonitrile in the mobile phase at a flow rate of 0.8 ml/min. The concentration of the mobile phase modifier (acetonitrile) increased

linearly from 0 to 50% over 125 min (0.4%/min). The main peaks that were present in 0-month and 4-month ripened cheese were isolated and purified by repeating the procedure 2 or 3 times with HPLC under the same conditions. When one peak was divided into 2 or 3 peaks by rechromatography of HPLC, the main peak was adopted as the peak for determination of the sequence. Purified peptides were evaporated, sealed under vacuum and hydrolyzed at 130°C for 3 hr with constant-boiling HCl. The amino acid composition of hydrolysate was analyzed with an automatic amino acid analyzer (Hitachi Model 835). Three amino acid sequences of each peptide were furthermore determined by an automatic protein sequencer (PSQ-1, Shimadzu Corp., Kyoto, Japan).

Results and Discussion

LMW peptides of unripe cheese and cheese ripened for 1 and 4 months were extracted and fractionated by HPLC. Fig. 1 shows the HPLC patterns for the cheese extracts from three different ripening periods. The patterns correlated with their ripening period, i.e., as the ripening period increased, the number and quantity of peptide peaks extracted increased. Therefore, the HPLC profiles of the extracts could provide valuable information on the ripening time of the cheese and is useful in monitoring the degree of cheese ripening.

Some of the peptides were isolated from unripe cheese and 4-month ripened cheese. The amino acid composition and N-terminal amino acid sequence of each peptide were examined, and the amino acid sequence of the purified peptides was determined.

The amino acid sequence of ten peaks was determined only in cheese ripened for 4 months. All peaks were identified as peptides, except for peaks 1, 2 and 5 which were amino acid corresponding to Tyr, Phe and Trp, respectively. Peaks 3, 4, 6, 7, 8, 9 and 10 were identified as α s1-CN(f1-9), α s1-CN(f1-7),

Fig. 1. HPLC profiles of 70% ethanol soluble extracts from the Gouda cheese.

A): unripe cheese, B): 1-month ripened cheese, C): 4-month ripened cheese, Dotted line: gradient profile of acetonitrile, Elution conditions were described in the text.

α s1-CN (f1-13), α s1-CN (f1-14), β -CN (f47-52), α s1-CN (f1-16), and α s1-CN (f1-17), respectively. Although some peaks of undetermined peptides were also purified with HPLC, their amino acid sequence could not be determined. It was especially difficult to purify the peaks between 80 min and 100 min of retention time with the described method for purification in this experiment. Peptides and amino acids which were identified from 4-month ripened cheese were also found in unripe cheese, except for α s1-CN (f1-7). Peaks 11 (Tyr), 12 (α s1-CN (f1-9)), 13 (Trp), 14 (α s1-CN (f1-13)) and 15 (α s1-CN (f1-16)) were considered the main peaks in unripe cheese. The peaks found in unripe

cheese increased in size with the lengthening of ripening period. However the peak corresponding to α s1-CN (f1-16) hardly increased.

Kaminogawa *et al.*⁴⁾ identified α s1-CN (f1-9), α s1-CN (f1-13), α s1-CN (f1-14) in the same Gouda-type cheese. We could, in addition to these peptides, isolate amino acids and peptides such as α s1-CN (f1-7), α s1-CN (f1-16), α s1-CN (f1-17), and β -CN (f47-52). Furthermore, the HPLC profiles obtained followed a clear pattern. It was considered that the use of ethanol might be suitable for extracting LMW peptides from cheese.

Recently, five peptides (α s1-CN (f1-9, f1-13, f11-?, f1-? and f1-?)²⁾ were found in Cheddar cheese. These peptides have been considered to be produced by the action of the lactococcal cell wall proteinase on the peptide α s1-CN (f1-23)³⁾ which is rapidly produced by chymosin²⁾. The (f1-9), (f1-13) and (f1-14) fragments of α s1-CN, which were identified in Gouda-type cheese, together with its (f1-7), (f1-18), (f1-20), and (f14-23) fragments, were isolated from a digest of α s1-CN (f1-23) made with a cell free extract from *Lactococcus lactis* subsp. *lactis* H 61. Furthermore, two proteases, designated as LEP-I and II, from the strain, were purified and characterized as enzymes related to the formation of these peptides^{7,8)}. In the experiment, all peptides identified except for β -CN (f47-52) were from the N-terminal region of α s1-CN. That is, these peptides belonged to α s1-CN (f1-23) which was produced by cleavage of the Phe23-Met24 bond of α s1-CN by chymosin during the initial stage of cheese making³⁾. It was, therefore, suggested that N-terminal peptides α s1-CN (f1-7), (f1-9), (f1-13), (f1-14), (f1-16) and (f1-17) of α s1-CN in the Gouda cheese in this experiment were also produced from α s1-CN (f1-23), which was formed by chymosin³⁾, by the action of protease from Lactococcal strain of starter bacteria in cheese. Hydrolysis of α s1-CN (f1-23) might be the important proteolysis for the formation and degradation of LMW peptides during cheese ripening.

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