생명과학회지
Korean J. Life Science, 6(1), 27-33, 1996

Characterization of Reactions Taken Place by A Mixed Culture of *Lactococcus lactis* Cells in Cheese Ripening

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Abstract

Reactions taken place by a mixed culture of *Lactococcus lactis* subsp. *cremoris* KH (lac^+prt^+) and KHA (lac^-prt^-) in cheese ripening have been investigated. Growth characteristics of the mixed culture showed commensalism, and the amounts of proteinases of the mixed culture were small enough. From these results, it is concluded that the production of bitter taste by the mixed culture is a small matter, even if the density of the mixed culture is highly maintained during cheese ripening. Hence, the mixed culture of KH and KHA cells could be a good cheese starter in accelerating the process of cheese ripening.

Introduction

Cheeses, such as Cheddar, are produced by storing the cheese block at 2-15℃ for several months[1]. During the process of ripening, the characteristic texture and flavor of different cheeses develop, since some remaining lactose are completely fermented and milk proteins are broken down to peptides and amino acids by starter cells. It is necessary to control the physicochemical changes taking place in the ripening process carefully, lest undesirable flavors and bitter taste develop[2].

Another motive for the introduction of starter cultures is the possibility of manipulation of the process of ripening. Since ripening processes take place over long period of time at low temperatures, a reduction of storage time during the ripening stage is considered very desirable. *Lactococcus lactis* subsp. *cremoris* (lac^+prt^+) cells are commonly used as starters in Cheddar cheese production. Attempts to reduce the ripening times have focused on getting high cell densities at the end of the fermentation phase. However, weak body and low calcium content in cheeses are caused by a rapid rate of acid production, at the same time, a high activity of proteinases has been associated with bitter taste in the final product[3]. Thus, mutants with reduced acid production capability have been sought.

Since prt^- (less proteolytic) mutants produce significantly less bitterness than the parent prt^+ cells, a mixture of lac^+prt^+ and lac^-prt^- (less proteolytic and non-lactose fermenting) cells is known to be a better starter culture than the prt^+lac^+ cells alone[4]. With the mixed cultures, synergistic interaction between the parent and the mutant cells occurs. Although a number of researchers[4-10] have studied the use of lac^-prt^- of *Lactococcal* cells in cheese manufacture and population dynamics of *Lactococcal* cells in milk fermentation, few have been

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reported the study of the mixed culture of *Lactococcus lactis* subsp. *cremoris* cells in cheese ripening. In this article, growth characteristics and proteinases of the mixed culture of *Lactococcus lactis* subsp. cremoris KH ($lac^+ prt^+$) and KHA ($lac^- prt^-$) cells are investigated.

Materials and Methods

Microorganisms

The strains of *Lactococcus lactis* subsp. *cremoris* KH and KHA were obtained from R. T. Marshall, Department of Food Science and Nutrition, University of Missouri, Columbia, Missouri, U.S.A. The lac^- strain KHA was isolated from lactose-fermenting KH strain after repeated transfers in new synthetic medium[11] containing 2μg ethidium bromide per ml[12]. Frequent tests with lactose-indicator agar showed that reversion of the phenotype is not significant in these strains.

The stock cells were maintained on an agar plate at 4℃. Subculturing was conducted monthly by transferring the cells into a sterile medium and inoculating at 30℃ for 18 h. Then the cells were poured on a new agar plate. Purity, morphology, and Gram reactions of the cultures were regularly checked using Gram staining of smears taken from agar plates as well as from culture broths.

Inocula were prepared by transferring 0.5mL of cell suspension into 9.5mL sterile media in test tubes and incubating at 30℃ for a period of 13－14 h. Five mililiters of this suspension were then transferred to 95mL sterile culture media in 500-mL flasks.

Medium composition

The cells were cultivated in the semi-synthetic medium[11] which contained, per liter of distilled water : lactose, 5.0g ; casein hydrolysate, 2.5g ; yeast extract, 0.95g ; NaH_2PO_4, 139.75mg ; $MgSO_4 \cdot 7H_2O$, 72mg ; $CaCl_2 \cdot 2H_2O$, 17.42mg ; ascorbic acid, 6.41mg ; nitric acid, 0.95mg ; $FeSO_4 \cdot 7H_2O$, 0.591mg ; and thiamine, 0.24mg. The lac^- mutant, KHA, was propagated in a modified medium in which glucose, instead of lactose, was added. Glucose and vitamines were separately autoclaved at 121℃ for 15min and mixed with other components of the medium at the operating temperature of 30℃ in order to avoid caramelization reactions.

The cells were spread on lactose indicator agar plates in order to check the ability of cultures to ferment lactose or their stability. The lactose indicator agar contained, per liter of distilled water : lactose, 5.0g ; agar, 15 g ; tryptone, 20.0g ; yeast extract, 5.0g ; gelatin, 2.5g ; sodium chloride, 4.8g ; sodium acetate, 1.5g ; and bromcresol purple, 40.0mg.

Analyses

Samples from the flasks were analyzed for the concentration of cells and lactate, and for the activity of proteinases. Coagulation was also examined in a tube of 10 %-reconstituted skim milk incubated at 30℃ for 1－2 days to check the phenotype of each cell. Cell concentration was determined by colony counting. With 0.1ml of the appropriate cell dilution, colonies were formed on a lactose indicator agar plate after incubated at 30℃ for 1－2 days. On the agar plate, lactose-fermenting colonies were yellow in contrast to the white non-lactose-fermenting variants. The concentration of lactate was measured using high-performance liquid chromatography with Polypore H column. A solution of 0.01 N H_2SO_4 was used as the mobile phase and pure helium gas was used to degas solvents. The elution flow rate was set at 0.2ml/min.

Proteinase activity in the culture broth was measured by using a 50 : 50 mixture of trypsin and chymotrypsin. The standard enzyme solution was prepared by dissolving equal amounts(20mg) of trypsin and chymotrypsin in 5ml of 0.001 N HCl and kept at 4℃ until use. A solution of substrate for the enzymes was prepared by adding 10g of whole casein to 1 liter of 50mM Na phosphate buffer solution (pH 7.8). The casein solution in the buffer was filtered through a 0.2m filter paper. The enzyme solution at different dilution was added to

the casein solution and incubated at 37℃. At various incubation times, 100μl of samples were withdrawn and added to 2.0ml OPA reagent[13] in a quartz cuvettes. These reaction mixtures were vortexed and allowed to react for 2－5 minutes at room temperature, and the absorbance was measured at 340nm. All data in figures represent average values for two replicates.

Results and Discussion

Preliminary experiments established the optimal pH for growth of KH and KHA cells as 6.5－7.0. Under these conditions, the cells showed the highest specific growth rate, as well as maximum cell density. Hence, 7.0 was chosen as the initial pH for the studies.

Growth characteristics of the mixed culture

Fig. 1 depicts lactate production by pure cultures and mixed cultures. Lac^- cells produced the smallest amount of lactate, and this mutant had an ability to produce lactate only by utilizing glucose. However, when lac^- cells were as paired with lac^+ cells, the mixed culture produced more acid than the sum of the acid produced by each lac^+ cells and lac^- cells individually, and it was less than the acid produced by $lac^+ + lac^+$ cells. These data suggest that there was a synergistic relationship between the acid producer KH and lac^- mutant, KHA cells. As expected, amounts of acid produced by $lac^+ + lac^-$ cells were not double that produced by lac^+ cells alone. This could be substrate limitation in acid production when bacterial cell numbers are raised to a critical concentration. Cells may thus have been limited in availability of lactose for utilization. This is supported by the bacterial colony counts given in Table 1.

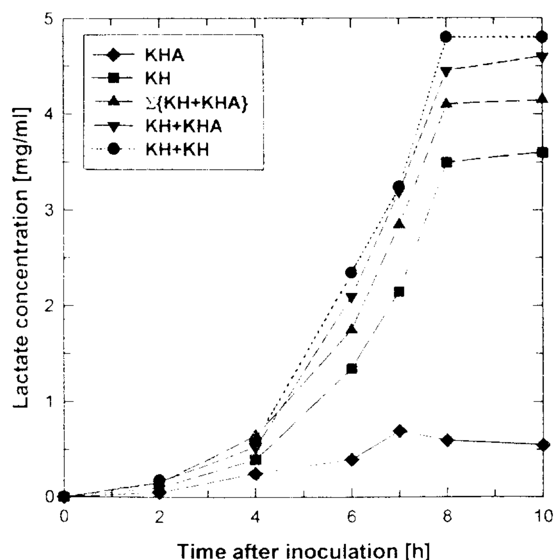

Fig. 1. Lactate production by pure cultures and mixed cultures.

Table 1. Total bacterial counts from colonies on lactose indicator agar

Treatment	Colony forming units per ml×10^5			
	0h	4h	8h	12h
KH	5	110	550	660
KHA	7	230	730	640
KH+KH	8	350	1260	1180
KH+KHA : KH	4	170	640	620
KHA	9	120	490	480

With the mixed starters, it has been known that commensalism occurs between the parent cells (lac^+prt^+) and its mutants (lac^-prt^-)[10]. Martley et al.[14] studied this interaction at comparable stages in acid production. It was found that the concentration of low molecular weight nitrogen compounds released in milk varied 15-fold among the observed strains of lactic Lactococci. Hence, it indicated that strains differ in their ability to support prt^- variants[15]. In mixed cultures enzyme specificity may also be involved since the number of different proteinases in the cell walls varies among strains.

It has been also reported that stimulatory interactions occur between prt^+ and prt^- variants in a single strain culture and between different strains in mixed culture[16,17]. For single strain cultures, the interaction takes place between different activity levels of proteinases. Since

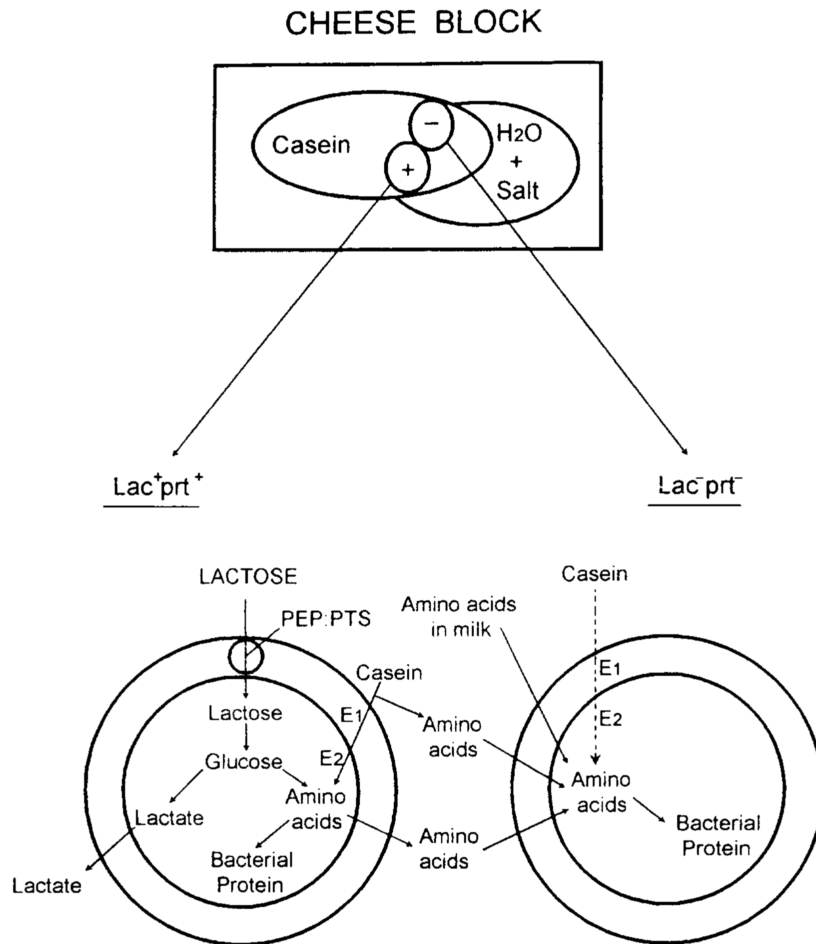

Fig. 2. Dynamics of commensalism in a mixed culture of cheese starters. E_1, proteinases ; E_2, peptidases ; PEP : PTS, phosphoenolpyruvate dependent phosphotransferase system ; unnected lines represent weak proteolysis by the enzymes.

prt$^+$ cells are known to donate some essential nitrogen nutrition for the growth of prt$^-$ cells, total cell population is affected by the proportion of prt$^+$ cells in a mixed culture without any important change in starter activity[3,18,19]. Keen[20] reported that some of the low molecular weight nitrogen compounds are free to diffuse away from the cell. When milk cultures of *Streptococcus lactis* were agitated, the cell growth rate decreased. It was thought that the products were more rapidly dispersed from the cell surface. In addition to the extracellular release of these peptides, it is presumed that some amino acids are produced internally after breakdown of peptides and flowed outside of the cell.

From all above information, the possible dynamics between the mixed culture of lac$^+$prt$^+$ and lac$^-$prt$^-$ cells occurring in the cheese block is depicted in Fig. 2. The cells are known to stay around the interface in which casein and moisture are present[21]. Lac$^-$prt$^-$ cells

cannot take amino acids by their microbial activity or proteolytic activity. Hence, they need low molecular weight amino acids for their growth. On the other hand, $lac^{-}prt^{-}$ cells can degrade lactose and casein to produce the amino acids. A mixed culture of $lac^{-}prt^{+}$ and $lac^{-}prt^{-}$ cells can survive as long as lactose medium remains in the cheese block.

Since weak body and low calcium content in the cheese are caused by a too rapid acid production, certain mutants which are slow acid producers can be particularly beneficial in cheese manufacture. However, the mutants have to be selected with low proteolytic activity and desirable dipeptidase activity. Otherwise, defective cheese may be produced. Thus, the number of normal starter bacteria cannot be increased without producing an atypical cheese. Therefore, lac^{-} mutants, along with regular cheese starter cultures, have an obvious potential in accelerated ripening methods.

Proteinase activity

The standard curve for the proteinase activities of the mixed culture in the culture broth was obtained by the use of Michaelis-Menten equation. The results are shown in Fig. 3. The standard curve showed that initial velocities of hydrolysis were linear with incubation time up to 5.0 mg of the enzymes per ml of 1% of whole casein.

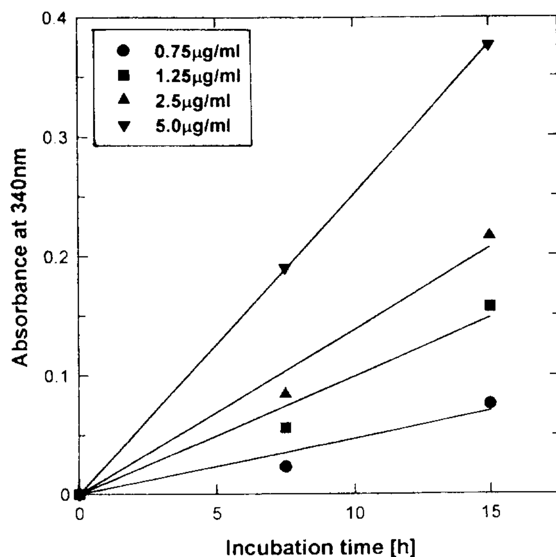

Fig. 3. Standard curve for the proteinase activity of KH in 1% whole casein.

Five ml of culture broth was sampled to measure proteinase activities of pure cultures and mixed cultures of KH and KHA cells. The activities of pure cultures and mixed cultures were very low, even not detectable in some cases. These results are in agreement with the report of Kamaly and Marth[17] that *Lactococcus cremoris* strains have inherently low proteinase activity. In addition, This could be because free amino acids present in the medium (owing to use of casein hydrolysate) suppress the production of the proteinases. Such an effect has been reported by Thomas and Pritchard[22].

Since high proteinase activity has been related to development of undesirable flavors[3] during the ripening of cheeses, it is not surprising that a cell line as desirable as *Lactococcus cremoris* has low proteinase activity. From the results, it is concluded that the mixed culture can be a good cheese starter.

Conclusions

Strain KHA ($lac^{-}prt^{-}$) cells did not grow at all on lactose. These cells produced less lactate on glucose and possessed low proteinase activity, suggesting the mixture of KH ($lac^{+}prt^{+}$) and KHA cells should be a good cheese starter.

Growth characteristics of the mixed culture showed commensalism. Some nitrogen compounds released as a result of proteolytic activity of KH cells were essential nutrition for the survival of KHA cells. The proteinase activity of the mixed culture was low enough not to produce bitter taste during cheese ripening. Now attempts to accelerate the process of ripening have focused on getting high cell density in the initial stage of cheese ripening, and the mixed culture of KH and KHA cells meets this requirement. In this respect, the mixed culture with proper ratio of each cell could be a good

cheese starter in accelerating cheese ripening.

Acknowledgement

This work was supported by '95 research grant to Research Center for Ocean Industrial Development from Korea Science and Engineering Foundation.

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초록 : 치즈숙성과정 중의 *Lactococcus lactis* 혼합균에 의하여 일어나는 반응들의 특성

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치즈숙성과정 중에서, *Lactococcus lactis* subsp. *cremoris* KH(lac^+part^+)와 KHA(lac^-prt^-)의 혼합균에 의하여 일어나는 반응을 연구하였다. 혼합균의 성장 특징은 한쪽 균의 도움에 의하여 같이 성장하는 특징을 나타내었으며, 이 혼합균의 단백질분해효소의 양은 충분히 적었다. 이 결과로부터 치즈숙성과정 중에 혼합균의 균밀도가 높게 유지되더라도, 이 혼합균에 의하여 생성되는 쓴맛의 생성량은 큰 문제가 되지 않는다는 결론을 얻을 수 있다. 그러므로, 치즈 숙성 과정을 촉진시키는데 있어서 KHAl의 혼합균은 좋은 치즈스타터균의 하나라고 할 수 있다.