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Characterization of Growth and Enzymes Produced by prt+lac+ and prt-lac- *Lactococcus lactis* Cells

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ABSTRACT

Lactococcus lactis subsp. *cremoris* strains KH (prt^+lac^+) and KHA (prt^-lac^-) are used as starter cultures in manufacture of ripened cheeses. The growth characteristics and the dipeptidases produced by these cells have been investigated in this study. A new semisynthetic medium was developed for growth of the cells. Growth on this medium was as good as or better than that on milk, the natural medium for cheese starters. Production of proteinases and dipeptidases by these cells was monitored. The effect of temperature on the activity and on the half-lives of dipeptidases was determined.

Index Entries: Cheddar cheese; dipeptidases; growth medium; temperature effect; half-life.

INTRODUCTION

The production of cheese starts with fermentation of pasteurized milk, during which coagulation occurs to form cheese curd. The curd is then cooked, whey is removed, and the cake is cheddared, salted, and pressed into a cheese block. Ripened cheeses, such as cheddar, are produced by storing the cheese block at 2–15 °C for several months (*1*). During the process of ripening, the characteristic texture and flavor of different

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cheeses develop. It is necessary to control the physicochemical changes taking place in the ripening process carefully, lest undesirable flavors and bitter taste develop (*2,3*). The physicochemical changes, in turn, are governed by the enzymatic content and the make-up of the microbial population present in the curd. As a result, starter cultures are often used in the fermentation of milk. Another motive for the introduction of starter cultures is the possibility of manipulation of the process of ripening. Since ripening processes take place over a long period of time at low temperatures, a reduction of storage time during the ripening stage is considered very desirable.

Lactococcus lactis subsp. *cremoris* (prt^+lac^+) 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. Since weak body and low calcium content in cheeses are caused by a rapid rate of acid production, mutants with reduced acid production capability have been sought. At the same time, a high activity of proteinases has been associated with bitter taste in the final product (*4*). Hence, prt^- mutants have been explored. These mutants produce significantly less bitterness than the parent prt^+ cells. Therefore, a mixture of prt^+lac^+ and prt^-lac^- cells is frequently a better starter culture than the prt^+lac^+ cells alone. With the mixed cultures, commensalism between the parent and the mutant cells occurs. A number of researchers have studied the proteinase-peptidase make-up of *Streptococcal* cells (*5–10*). Few published studies have reported on the peptidase make-up of the *Lactococcus cremoris* cells, and even fewer on the effect of growth conditions on the enzymes. In this article, the results of a study of fermentation characteristics of the lac^+ and lac^- cells of *Lactococcus lactis* subsp. *cremoris* and the peptidases of these strains are presented.

MATERIALS AND METHODS

Microorganisms

The strains of *Lactococcus lactis* subsp. *cremoris* KH and KHA were obtained from T. R. Klaenhammer, Department of Food Science, North Carolina State University, Raleigh, NC. The characteristics of these strains have been investigated by Wijayaweera (*11*) and Abu-Tarboush (*12*). The lac^- strain KHA was isolated from lactose-fermenting KH strain after repeated transfers in M17-glucose medium containing ethidium bromide or acriflavin hydrochloride (*13*). The proteolytic activity of strain KHA is significantly less than that of the strain KH (*11*). Accordingly, strain KH has been designated as lac^+prt^+ and the strain KHA as lac^-prt^-. Frequent tests with lactose-indicator agar showed that reversion of the phenotype is not significant in these strains.

The stock cells were maintained in liquid broth at 4°C. Subculturing was conducted weekly by transferring the cells into sterile medium and inoculating at 30°C for 24 h. 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 0.5 mL of cell suspension into 9.5 mL sterile media in test tubes and incubating at 30°C for a period of 14–15 h. Five milliliters of this suspension were then transferred to 95 mL sterile culture media in 500-mL Erlenmeyer flasks and incubated for 8–9.5 h at 30°C.

Fermentations were conducted in a 1.5-L fermenter (Omni-culture fermenter, Virtis, Gardiner, NY) with 1 L working volume. Speed of rotation was controlled at 150 rpm, temperature at 30°C, and pH at 7.0 by automatic addition of 1.0*N* NaOH.

Medium Composition

Lactococci are generally cultivated in milk or in modified M17 medium (*14*). Both provide for the complex nutritional requirements of the cells. Use of these media resulted in complications in the analyses of samples for the fermentation products, especially the acids. Hence, a new semisynthetic medium was developed on the basis of the nutritional requirements of *Lactobacillus* and *Lactococcus* cells (*15*) and the composition of milk. This semisynthetic medium consisted of, per liter of distilled water: lactose, 5.0 g; Casein hydrolysate, 2.5 g; yeast extract, 0.95 g; NaH_2PO_4, 139.75 mg; $MgSO_4 \cdot 7H_2O$, 72 mg; $CaCl_2 \cdot 2H_2O$, 17.42 mg; ascorbic acid, 6.41 mg; nicotinic acid, 0.95 mg; $FeSO_4 \cdot 7H_2O$, 0.591 mg; and thiamine, 0.24 mg. Use of this semisynthetic medium permitted analysis of samples by liquid chromatography and resulted in growth of cells that was as good as or better than that in the modified M17 medium. The lac^- mutant, KHA, was propagated in a modified medium in which glucose, instead of lactose, was added. Glucose and vitamins were separately autoclaved at 121°C for 15 min and mixed with the other components of the medium at the operating temperature of 30°C, in order to avoid caramelization reactions.

Analyses

Samples from the fermenter were analyzed for the concentration of cells, sugars (lactose/glucose), lactic acid, and for activities of proteinases and selected peptidases. Cell concentration was monitored by measuring the absorbance at 410 nm. Cell concentrations were also determined as cell dry wt/L after drying the cells in an oven overnight at 110°C and by colony counting. The concentrations of sugars and of lactic acid were determined using an isocratic HPLC procedure, described by Tu et al. (*16*).

Proteinase activity in the culture broth was measured by the method of Church et al. (*17*) with casein as the substrate. Measurements from samples out of a sterile flask containing culture medium served as control. Total intracellular protein content was measured using the Bradford method (*18*) after disruption of cells using ultrasound. Dipeptidase activity was measured in cell-free extract according to the method of Abu-Tarboush (*12*) with four dipeptides (arg-gly, gly-tyr, leu-gly, and val-leu) as substrates.

RESULTS AND DISCUSSION

Preliminary experiments established the optimal pH for growth of KH and KHA cells as 6.5–7.0. This pH range is also the one used in fermentation of milk during production of ripened cheeses. Under these conditions, the cells showed the highest specific growth rate, as well as maximum cell density. Hence, a pH of 7.0 was chosen for the studies.

Growth Characteristics of KH and KHA Cells

Bath fermentations were conducted in a pH-controlled, unaerated stirred-tank bioreactor. Typical profiles for growth of KH and KHA cells on glucose containing semisynthetic medium are shown in Figs. 1 and 2. KHA cells did not grow on lactose medium even after 5 d, but showed a rapid growth on glucose. KH cells grew equally well on lactose as well as glucose. In all the cases, the cell concentrations decreased at the end of growth owing to cell lysis. This phenomenon has been commonly observed for starter cultures in cheese making (*19*). Key reactions in cheese ripening are carried out by the enzymes that are either intracellular or on the surface of the cells (*20,21*). Cell lysis provides a mechanism for release of the enzymes into the cheese matrix.

Table 1 shows the calculated maximum specific growth rates from these studies. The error bounds shown in the table were calculated from duplicate experiments and suggest a high degree of reproducibility of data. Also shown in the same table are maximum specific growth rates for some other strains of *Lactococcus lactis* subsp. *cremoris* as reported in literature. Although the value of μ_m varies with the strains, this table clearly demonstrates that the semisynthetic medium can support cell growth as well as milk, the natural medium. The maximum specific growth rates of KH and KHA cells found in our studies were on the upper end of the values reported for other strains in milk. Strain KH is closely related to *Lactococcus lactis* subsp. *cremoris* HP and WG_2. The specific growth rates of KH and KHA cells should be similar to the μ_m value of these strains. In Table 1, the μ_m of KH and KHA cells is some what higher, an effect that can be ascribed to the presence of casein hydrolysate in the modified medium used in the present study. For growth on

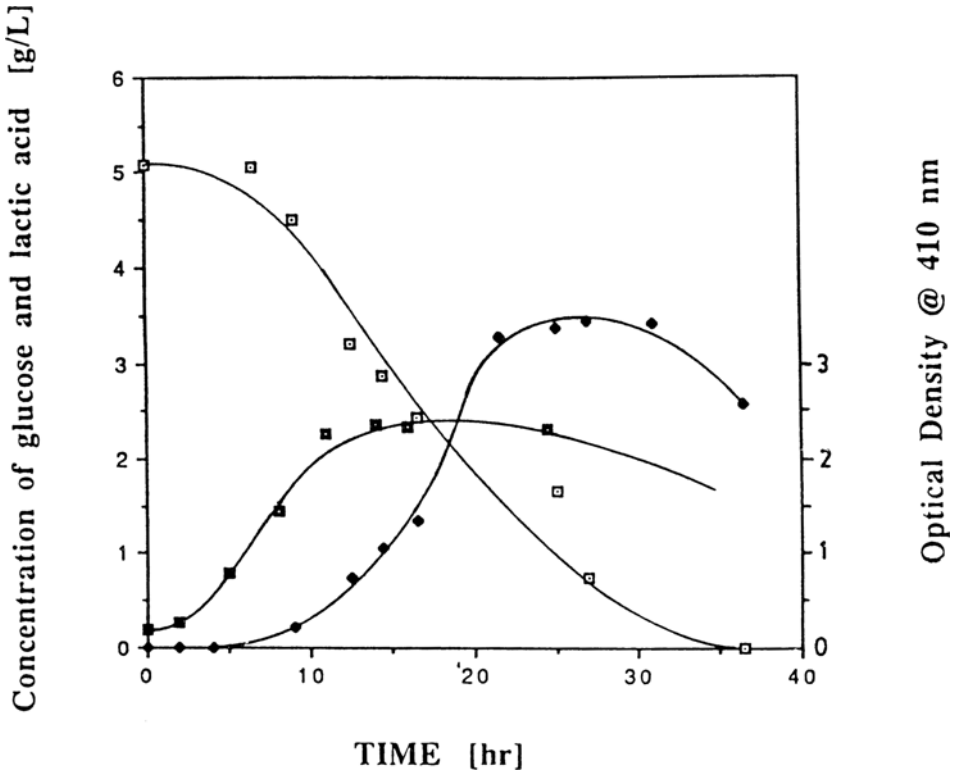

Fig. 1. Growth of *Lactococcus cremoris* KH cells on glucose at pH 7.0. □: glucose; ◆: lactic acid; ■: optical density.

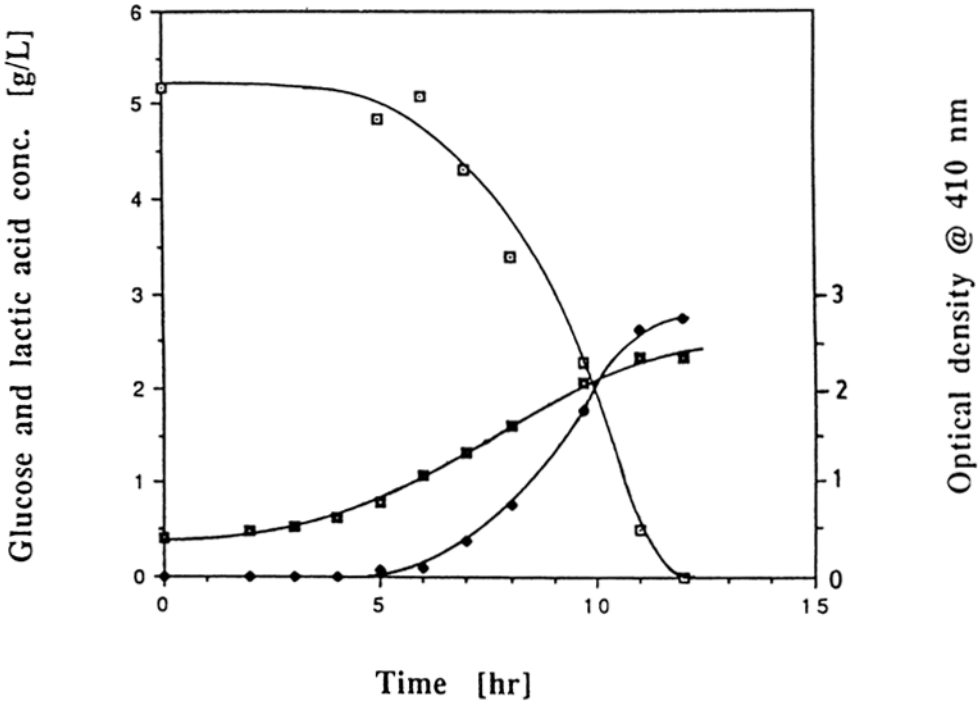

Fig. 2. Growth of *Lactococcus cremoris* KHA cells on glucose at pH 7.0. □: glucose; ◆: lactic acid; ■: optical density.

Table 1
Maximum Specific Growth Rates of *Lactococcus cremoris* Strains

Strain	Substrate	μ_m, h^{-1}	Source
KH	Lactose	0.57±0.04	
KH	Glucose	0.54±0.05	This study
KHA	Glucose	0.55±0.03	
HP	Milk	0.39	
WG_2	Milk	0.35	
E_8	Milk	0.51	ref. (22)
ML_1	Milk	0.52	
AM_1	Milk	0.45	

milk, the proteinase activity of starter cells plays an important role in supply of low-molecular-weight nitrogeneous compounds needed for cell growth (*23*); prt⁻ cells, which have a low surfacebound proteinase activity, are limited by the supply of low-molecular-weight in amino acids and peptides, and have a lower growth rate in milk. In the semisynthetic medium used in this study, casein hydrolysate provides a ready supply of amino acids and peptides for prt⁻ cells, and no significant difference was observed between the growth rates of prt⁺ and prt⁻ cells.

Proteolytic Activity

Proteinase activity in the cell suspension was measured, according to the methods described by Church et al. (*17*). This method involves adding cell suspension to a solution of trichloroacetic acid, filtering, and reacting the filtrate with *o*-phthaldialdehyse (OPA) reagent, followed by measuring the absorbance at 340 nm. With this method, very low proteolytic activity was measured in samples collected from pH-controlled fermentations involving KH or KHA cells. There are two likely reasons for this observation: (1) The strains have inherently low proteolytic capability. (2) Free amino acids present in the medium (owing to use of casein hydrolysate) suppress the production of proteinases. Such an effect has been reported by Thomas and Pritchard (*9*). A third possible reason for this effect could be the influence of Ca^{2+} ions. Low concentrations of Ca^{2+} ions have been related to a reduction of the rate of release of proteinases into solution (*9*). However, at a concentration of Ca^{2+} ions above $10^{-6}M$, this effect has not been observed (*22*). In the semisynthetic medium used in this study, the concentration of Ca^{2+} ions was $> 10^{-6}M$. Therefore, the effect of calcium was probably insignificant.

In order to study the above two effects further, both the strains (KH and KHA) were grown in 10% reconstituted nonfat milk, and proteinase activities were measured during the experiment. These data have been shown in Fig. 3 and 4. The controls belong to samples from parallel sterile

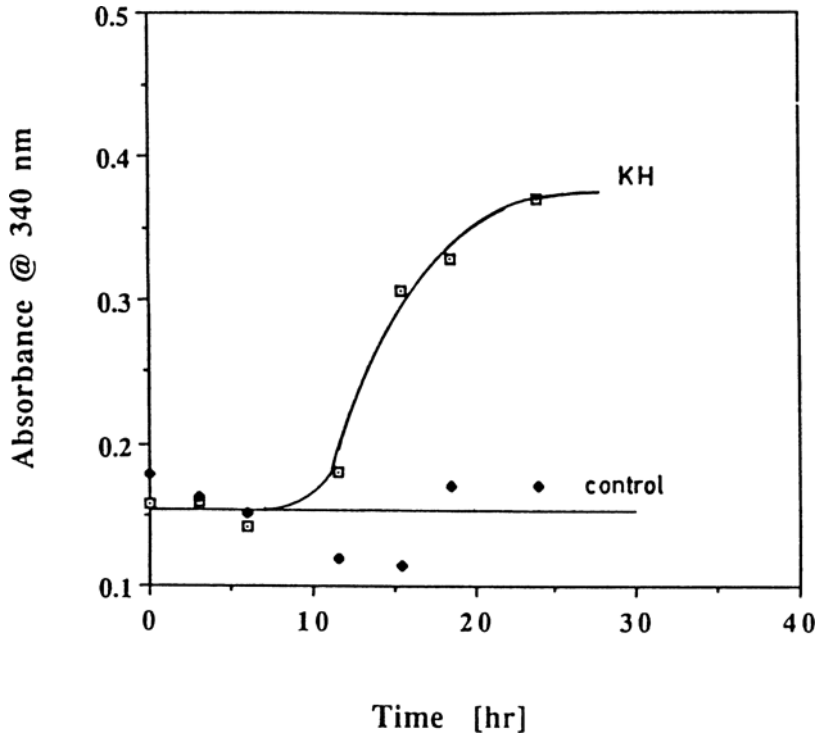

Fig. 3. Proteolytic activity in fermentation broth of KH cells.

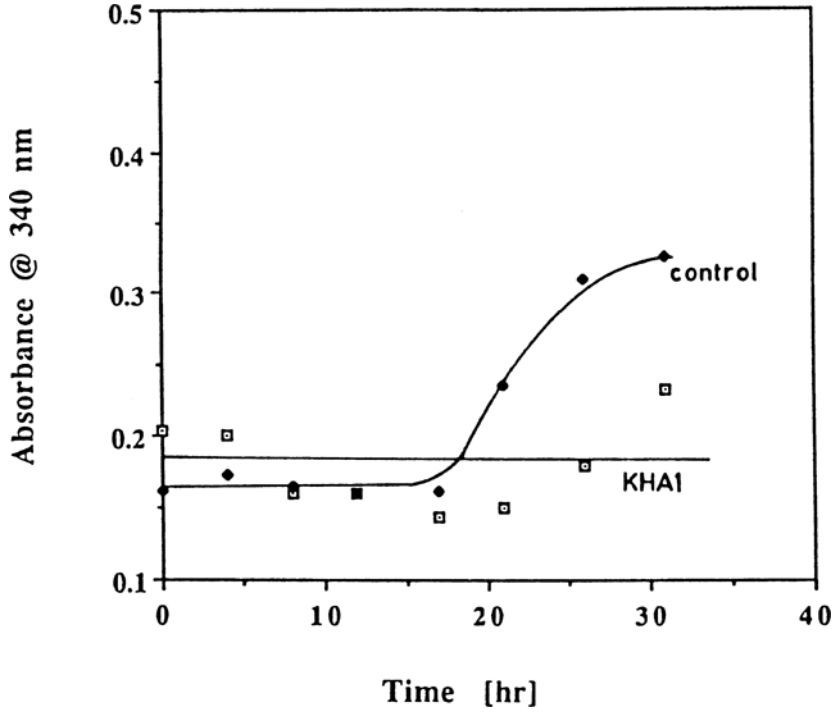

Fig. 4. Proteolytic activity in fermentation broth of KHA cells.

flasks that were not inoculated with cells. On the whole, KH cells showed considerably higher activity than KHA cells, even though the activities are very low. These results are in agreement with the reports of Church et al. (*17*) and Kamaly and Marth (*24*) that *Lactococcus cremoris* strains have inherently low proteinase activity. Similar activities were also reported by Sing and Klaenhammer (*13*). The activities shown in Fig. 3 are similar to those reported for *Lactococcus cremoris* strains HP and WG_2. During the ripening of cheeses, high proteinase activity has been related to development of undesirable flavors (*4*). Hence, it is not surprising that a cell line as desirable as *Lactococcus cremoris* has low proteinase activity. In consumer taste tests, cheddar cheese ripened over 9 mo has been preferred over one 6-mo-old cheese. This, however, results in longer periods required for ripening of the cheeses.

Dipeptidase Activity

Intracellular dipeptidase activity in the KH and KHA cells for four dipeptides (arg-gly, val-leu, leu-gly, and gly-tyr) was measured in samples collected from controlled-pH experiments at different fermentation times. For each sample, the measurements were conducted in quadruplicate, and the average data have been plotted in Figs. 5–7 as volumetric activity. In these figures, optical density and total protein profiles have also been plotted for the sake of reference. In none of the cases has any activity against dipeptide gly-tyr been observed. In general, the production of dipeptidases appeared to be growth related and increased with increase in cell density. Lysis of cells resulted in reduction in dipeptidase activity also. Specific dipeptidase activity (defined as μmol/mg protein/min) was maximum in the exponential growth phase, and declined in the late log and stationary phases of growth (data not reported).

Comparison of Figs. 6 and 7 also suggests that both the strains had similar activity of dipeptidases while growing on glucose. For strain KH, dipeptidase activities while growing on glucose were considerably higher than those in cells growing on lactose. This is a rather puzzling observation, since the maximum specific growth rate as well as the maximum cell density of KH cells are basically the same on the two substrates. For each of the experiments reported here, a number of replicates were made, and a good reproducibility was observed.

Effect of Reaction Temperature on the Activities of Dipeptidases

During the late log phase of an experiment involving controlled-pH growth of KHA cells on glucose, a large cell sample was collected, and the activities of dipeptidases were measured at different temperatures. These

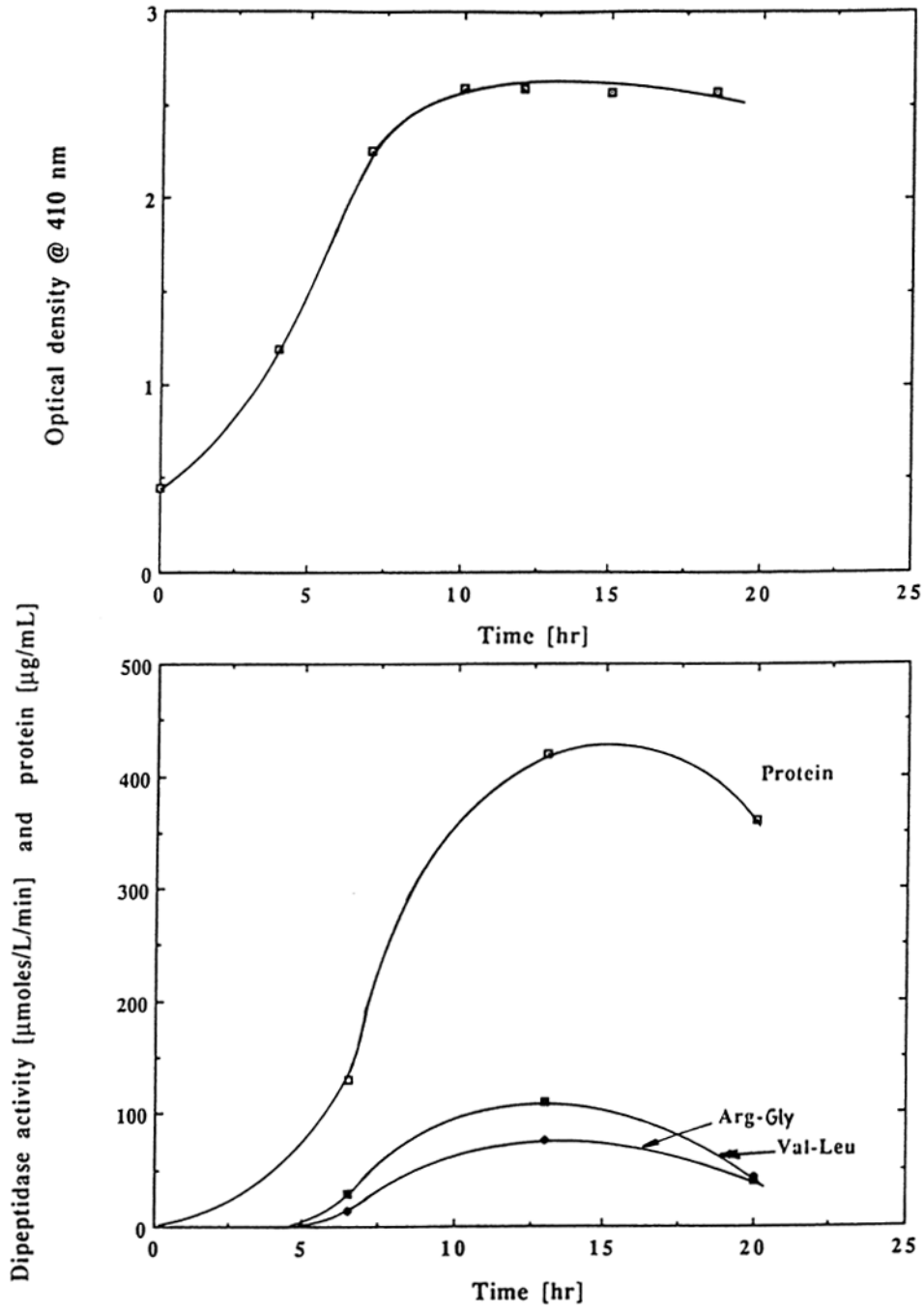

Fig. 5. Intracellular dipeptidase activity, in KH cells grown on lactose, for different dipeptides.

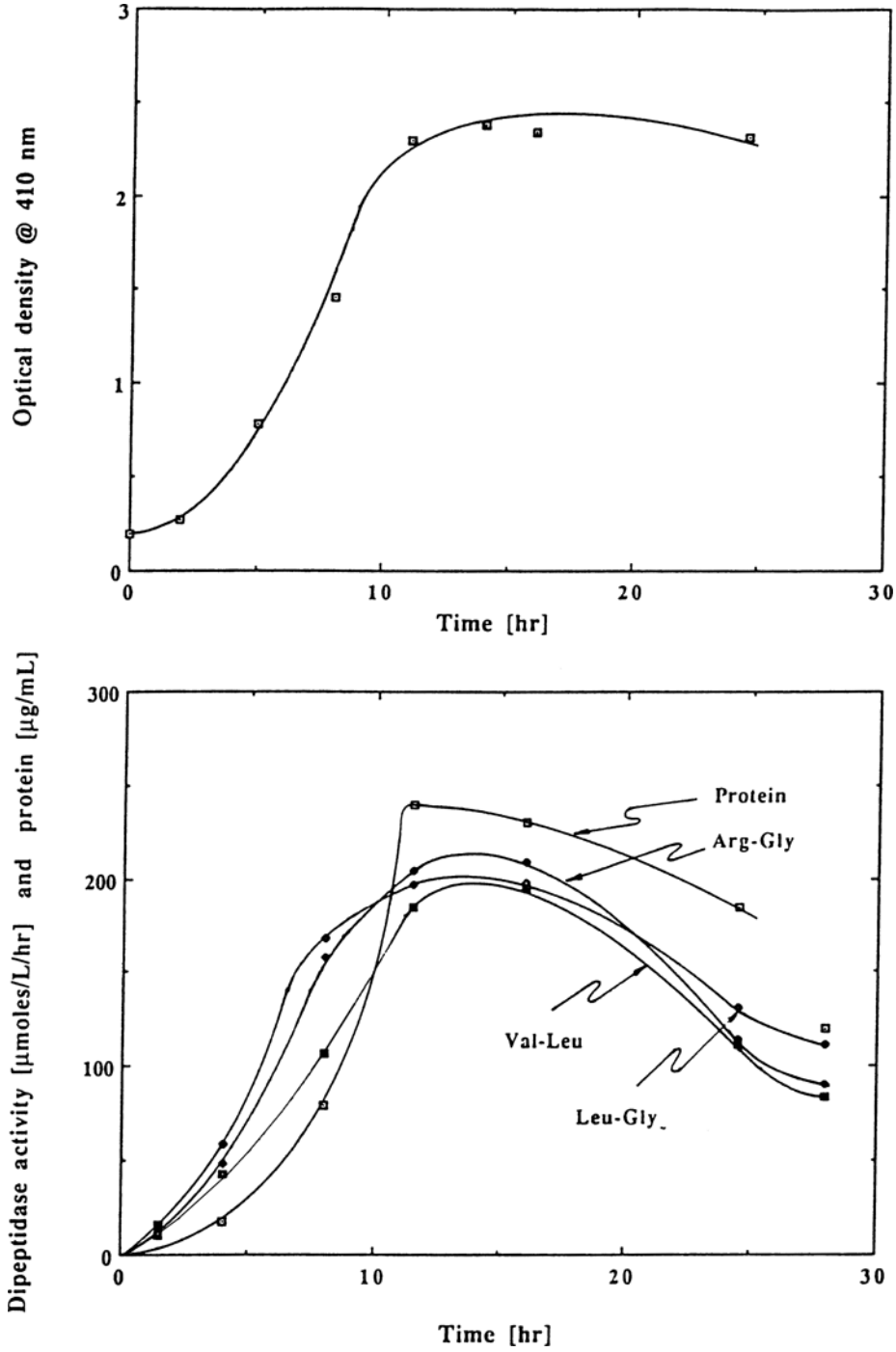

Fig. 6. Intracellular dipeptidase activity, in KH cells grown on glucose, for different dipeptides.

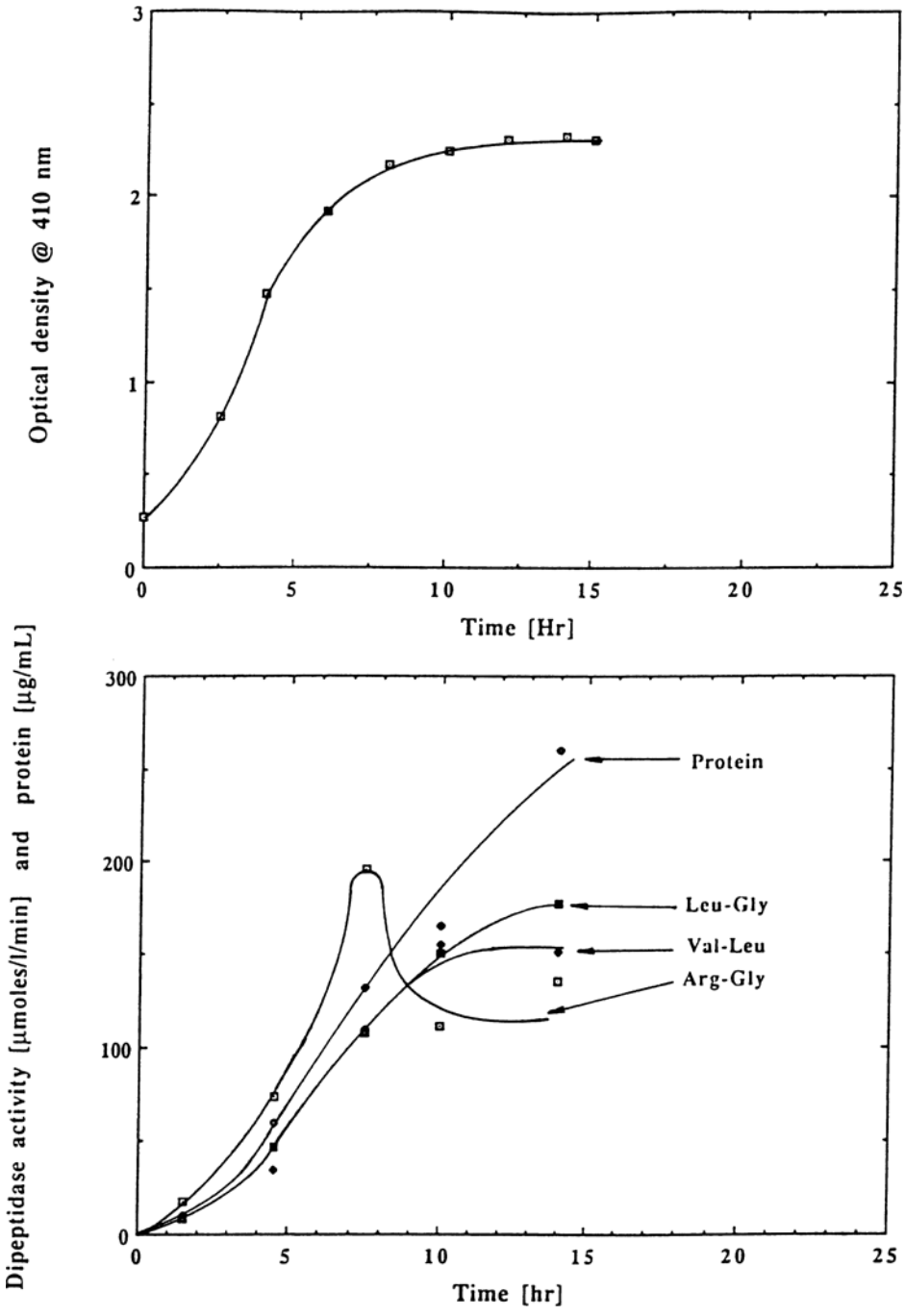

Fig. 7. Intracellular dipeptidase activity, in KHA cells grown on glucose, for different dipeptides.

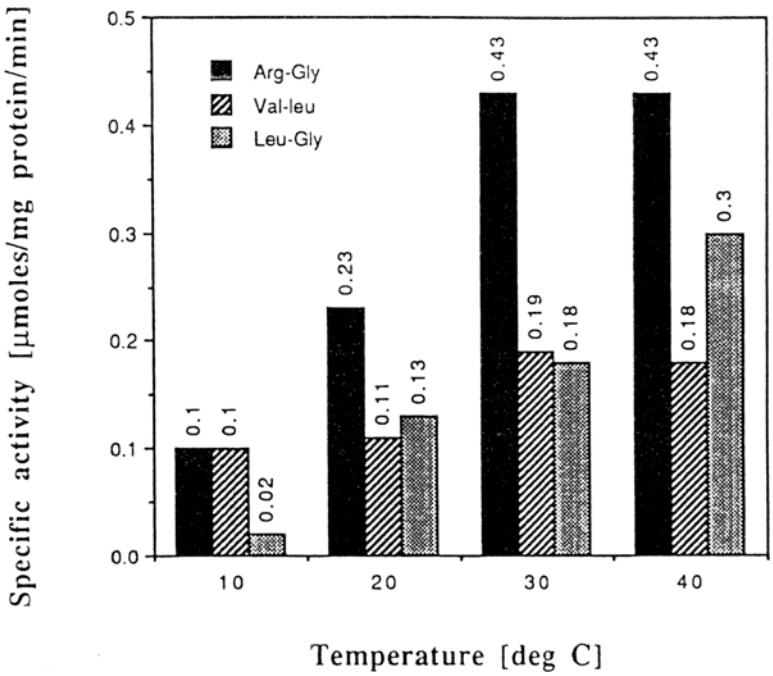

Fig. 8. Effect of reaction temperature on dipeptidase activity in *Lactococcus cremoris* cells.

data have been presented in Fig. 8 in the form of a bar chart. The measurements were made in duplicate and were highly reproducible. In general, the activities increased with increase in temperature, up to 40°C. These results are in agreement with the results reported in literature (*12,19*). The temperature of 30°C suggested by Law et al. (*19*) was found to be acceptable. The temperature effect on the enzymatic activities for different substrates showed substantial variation. The activity against Leu-gly steadily increased by an order of magnitude between 10–40°C. On the other hand, the activity against val-leu showed the smallest increase. Arrhenius plots of these data resulted in the following values of activation energies for the different enzymes:

val-leu	2.14×10^4kJ/kmol
arg-gly	4.00×10^4kJ/kmol
leu-gly	6.74×10^4kJ/kmol

Effect of Temperature on the Half-Lives of Dipeptidases

The crude cell-free extracts of KHA cells were stored at different temperatures for different periods of time, and the activities against dipeptide leu-gly were measured at 30°C. The results have been presented in Fig. 9

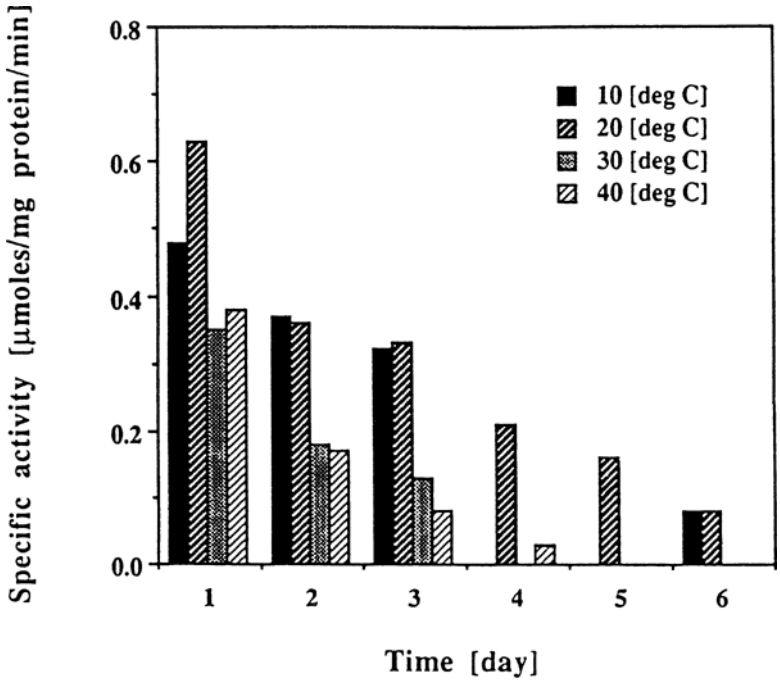

Fig. 9. Effect of temperature on half-life of dipeptidases in cell-free extracts of *Lactococcus cremoris*.

and show that the rate of inactivation of the enzyme increases rapidly with temperature. The first-order decay rate constants calculated from these data are:

10°C	0.35 hr^{-1}
20°C	0.43 hr^{-1}
30°C	0.55 hr^{-1}
40°C	0.63 hr^{-1}

Similar results were also obtained for the activity against arg-gly.

The rates of deactivation obtained here are higher than those obtained by Law et al. (*19*), who measured their enzyme activity after gel filtration on Sephadex G-200. It is not clear whether proteinases play a role in deactivation of the dipeptidases.

CONCLUSIONS

The maximum specific growth rates of *Lactococcus cremoris* KH and KHA cells were almost the same on glucose and on lactose. Strain KHA did not grow at all on lactose, confirming its lac^- character. These cells possessed relatively low proteinase activity, suggesting that the mixture

of KH and KHA cells should form a good starter culture for manufacture of ripened cheeses. Dipeptidase activities of these two strains were similar, but there was some specificity for different dipeptides. The dipeptidase activity increased steadily with cell growth, although the specific activity decreased after exponential growth phase. The activity was highest at 40°C, but the enzymes were most stable at low temperatures.

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