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Chemical Conversion of α-Keto Acids in Relation to Flavor Formation in Fermented Foods

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Formation of flavor compounds from branched-chain α-keto acids in fermented foods such as cheese is believed to be mainly an enzymatic process, while the conversion of phenyl pyruvic acid, which is derived from phenylalanine, also proceeds chemically. In this research, the chemical conversion of α-keto acids to aldehydes with strong flavor characteristics was studied, with the main focus on the conversion of α-ketoisocaproic acid to the aldehyde 2-methylpropanal, and a manganese-catalyzed reaction mechanism is proposed for this conversion. The mechanism involves keto−enol tautomerism, enabling molecular oxygen to react with the β-carbon atom of the α-keto acid, resulting in a peroxide. This peroxide can react in several ways, leading to unstable dioxylactone or noncyclic intermediates. These intermediates will break down into an aldehyde and oxalate or carbon oxides (CO and CO_2). All the α-keto acids tested were converted at pH 5.5 and in the presence of manganese, although their conversion rates were rather diverse. This chemical reaction might provide new ways for controlling cheese flavor formation with the aim of acceleration of the ripening process or diversification of the flavor characteristics.

KEYWORDS: α-Keto acids; 2-oxo acids; flavor; leucine; aroma formation; 2-methylpropanal; dairy; cheese

INTRODUCTION

Many flavors in fermented products are derived from amino acids. Examples of important amino-acid-derived flavor compounds in cheese are aldehydes such as benzaldehyde, 2-methylpropanal and 3- and 2-methylbutanal, but also methionine-derived sulfur compounds, such as methanethiol and DMDS (*1*−*4*). Most flavor forming reactions in fermented products such as cheese are enzymatic (*5*−*9*). Protein degradation is initiated by proteolysis and peptidolysis, leading to free amino acids (*10*). The amino acids are mostly enzymatically transaminated in the bacterial cell to the corresponding α-keto acid (*11*, *12*). These α-keto acids can be converted to various metabolites, such as the aldehydes and sulfur compounds mentioned above. The α-keto acid of leucine (KICA) for example, can be enzymatically decarboxylated by a number of *Lactococcus lactis* strains to 3-methylbutanal (*13*, *14*). Although most of these reactions are enzymatic, the α-keto acids of phenylalanine (phenylpyruvic acid = PPA) and methionine (KMBA) are also chemically converted to flavor compounds such as benzaldehyde and methylthioacetaldehyde. The occurrence of the chemical conversion of PPA was demonstrated by Villablanca et al. (*15*) and by Nierop Groot et al. (*16*), and the conversion of KMBA to methylthioacetaldehyde has been identified by Yvon et al. (personal communication). The conversion of phenylpyruvic acid is due to oxidation by molecular oxygen and is catalyzed by divalent cations such as manganese (*15*, *16*).

The reactivity of ketones such as α-keto acids is mainly due to the existence of their enol tautomers (*17*−*19*). Molecular oxygen is able to react with the enol, leading to the formation of a peroxide on the β-carbon atom (*20*). The nature of this peroxidation is not totally clear and might as well proceed directly as via a radical mechanism (*20*, *21*). The very reactive peroxide intermediary of *p*-methoxyphenylpyruvate can react with the acid or keto carbon atom of the molecule, resulting in a dioxylactone or dioxyethanol, although the latter reaction only occurs under basic, non nucleophilic conditions (*22*). The unstable dioxylactone formed from *p*-methoxyphenylpyruvate will decompose into an aldehyde and the carbon oxides (CO_2 and CO) (*22*). A hydrophilic solvent, like water, is also able to react with the keto function of the peroxide intermediate, resulting in a noncyclic intermediate (*22*). Oxalic acid and water can be split off, leaving an aldehyde. To summarize, the conversion of several keto acids depends strongly on the circumstances (e.g., solvent and pH), but the products of these reactions are an aldehyde and oxalate or carbon oxides.

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This research focuses on revealing the characteristics and reaction mechanism of the chemical conversion of α-keto acids, leading to compounds relevant for fermented foods. It is important to note that the conversion of substrates present in the food takes place under mild conditions. The branched chain α-keto acid of leucine, α-ketoisocaproic acid, was chosen for this study because its conversion product, 2-methylpropanal, has an important flavor impact in foods.

MATERIALS AND METHODS

The Chemical Conversion was assayed in a 20-mL headspace vial with a reaction volume of 5 mL or in 1 mL HPLC vials, with a reaction volume of 0.5 mL. Final concentrations of reactants in the reaction mixture were 50 mM buffer, 10 mM metal chloride salt, 10 mM substrate. Standard conditions were succinate buffer (pH 5.5), manganese chloride, and α-ketoisocaproic acid as substrate. Buffers were succinate (pH 3.5−6.5), glycerolphosphate (pH 4.5−7.2), bis-Tris-propane (pH 6.4−7.4), HEPES (pH 6.4−7.8), and Tris-HCl (pH 7−8). Metal salts: $MnCl_2$, $MnSO_4$, $MgCl_2$, $CaCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, $CoCl_2$, $ZnCl_2$, and NaCl. Substrates used in this study were α-ketoisocaproic acid (KICA), 2-oxo-3-methyl pentanoic acid, α-ketoisovaleric acid, α-ketohexanoic acid, α-ketopentanoic acid, α-ketobutanoic acid, pyruvic acid, 4-methylthio-2-oxobutanoic acid (KMBA), phenylpyruvic acid (PPA), 3-(4-hydroxyphenyl)-2-oxopropanoic acid, 3-indol-3-yl-2-oxopropanoic acid (indole pyruvate), 2-oxopentanedioic acid, 4-methylpentanoic acid, 3-methylbutanoic acid, and 2-methylpropanoic acid. All substrates were analytical grade and obtained from Sigma (Zwijndrecht, The Netherlands) or Fisher (Landsmeer, The Netherlands). At $t = 0$, substrate was added to the rest of the reaction mixture, and the vial was closed (including 15 mL air in the headspace), and placed in a incubator which was shaking at 500 rpm for 10 s on/5 s off intervals at 40 °C (Combi Pal, CTC Analytics, Zwingen, Switzerland).

Direct Inlet Mass Spectrometry (DI-MS) was used for the on-line monitoring of product formation. The method used was a slightly adapted version of the method published previously (*14*). At regular intervals, a 150-μL headspace sample was taken from the incubator. The sample was injected with a 1.0-mL syringe at 60 °C and 20 μL/s by the Combi Pal auto sampler (CTC Analytics, Zwingen, Switzerland) into the gas chromatograph (CE-Instruments, Milan, Italy). The GC was equipped with an 8-m × 0.1-mm deactivated silica column (Interscience, Breda, The Netherlands). Helium was used as carrier gas, with a column flow of 2.5 mL/min and split flow of 10 mL/min. The oven temperature was 150 °C. Single ions at $m/z = 58$, 72, and 105 were recorded by a quadrupole mass spectrometer (Trace MS, CE-Instruments, Milan, Italy). These specific *m*/*z* values represent fragments of 3-methylbutanal, 2-methylpropanal, and benzaldehyde, respectively. Acquisition time was 30 s. The height of the response at a given *m*/*z* value is directly correlated to the concentration of the compound. Quantification was done by using a calibration curve ranging from 10 to 1000 μM in the same buffer as that used in the experiments, which was analyzed before and after each experiment in triplicate. The conversion rate is defined as the change of concentration in time, expressed in μM/h.

High-Pressure Liquid Chromatography. HPLC was used for the determination of α-keto acids and organic acids. The reaction mixture was prepared as described above and incubated in a model 717 auto sampler (Waters, Milford, MA) at 40 °C. At regular intervals, samples (25 μL) were taken and injected to the system. For the analysis of polar molecules (pyruvic acid, 2-oxobutyric acid, 2-oxopentanedioic acid, and oxalate) the system consisted of a LC-10AT pump (Shimadzu, Tokyo, Japan), pumping 0.01 M methane sulfonic acid with an isocratic flow of 0.6 mL/min over a Rezex Fast Fruit precolumn (100- × 7.8-mm) and a double Rezex Organic Acid column (300- × 7.8-mm, Phenomenex, Torrance, Ca) at 30 °C. The other acids were analyzed using the same system, but equipped with a wide pore C_{18}−RP column (250- × 4.6-mm, Bio-Rad, Veenendaal, The Netherlands), and components were eluted at 40 °C with a gradient from 5 to 30% acetonitrile in demineralized water containing 0.1% trifluoracetic acid (TFA). A model 1481 Lamda-max detector (Waters, Milford, MA) was used at 220 nm to detect the compounds in both system configurations. Concentrations were calculated using separate calibration samples containing 4, 6, 8, and 10 mM of the acids in 50 mM succinate buffer (pH = 5.5).

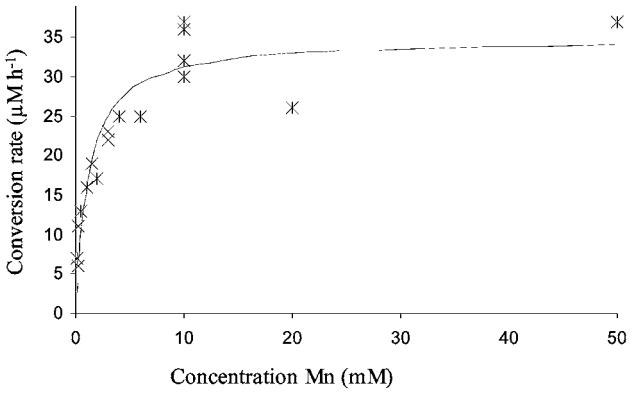


Figure 1. Effect of manganese concentrations on the conversion rate of KICA to 2-methylpropanal (succinate buffer, pH 6.0).

Gas Chromatography. GC was used to determine the concentrations of O_2 and CO_2 in the headspace. The reaction mixtures were prepared as described above and incubated at 40 °C. Before each measurement, samples were inverted four times and cooled to 25 °C. For 5 s, a headspace sample was taken and analyzed on a CP2001 gas chromatograph (Chrompack, Middelburg, The Netherlands), equipped with a HayeSep A column (25 cm) and a MS 5A column (4 m). Because the sampled volume was about 0.5 mL, and samples were analyzed in quadruplicate, a separate reaction vial was used each time interval. Concentrations were calculated by using air (21% O_2, 0.033% CO_2) and a calibration gas (1.00% CO_2, 1.00% O_2) for calibration.

RESULTS AND DISCUSSION

The production of branched chain aldehydes from amino acids in cheese is believed to be a two step process consisting of a transaminating step and decarboxylating step. Leucine, the most abundant branched-chain amino acid in cheese, is transaminated to α-ketoisocaproic acid (KICA), which can subsequently be decarboxylated to 3-methylbutanal (*13*). Under certain conditions, fermented dairy products had unexpectedly high 2-methylpropanal concentrations (unpublished data). This flavor compound is believed to be produced from valine via an enzymatic pathway similar to the one elucidated for leucine, with α-ketoisovaleric acid as the intermediate (*5*−*7*). To control cheese flavor formation, it is important to understand the characteristics of these enzymatic conversions. Studying the enzymatic decarboxylation of KICA by static headspace measurements (*13*) of incubations of cell free extract (CFE) of *Lactococcus lactis* B1157 with KICA revealed that the concentration of 2-methylpropanal also increased (data not shown) although no α-ketoisovaleric acid was added to these incubations. Repetition of the experiment with cooked CFE, thereby inactivating the decarboxylating enzyme, still led to the same increase of 2-methylpropanal concentration. This suggested that KICA could also be converted chemically with 2-methylpropanal as one of the products. This reaction was studied in more detail using direct inlet mass spectrometry.

None of the tested metal-salts, except the mangenese-salts, were able to catalyze the reaction at a concentration of 10 mM either at pH 5.5 or pH 7.5. Addition of 10 and 20 mM EDTA to reaction mixtures containing 10 mM $MnCl_2$ resulted in a decrease in the conversion rate to 12 and 4% respectively, indicating the need for free manganese ions. The correlation

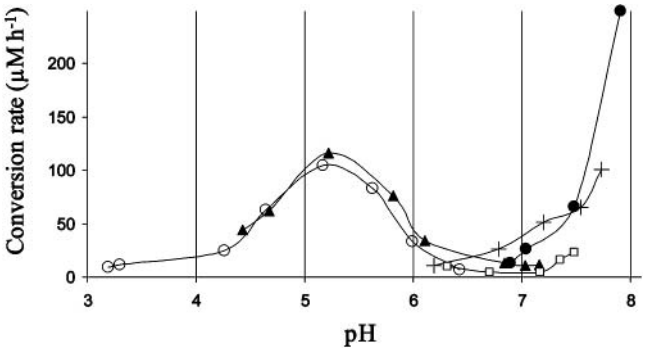


Figure 2. Effect of pH on the conversion rate of KICA to 2-methylpropanal. (10 mM KICA, 10 mM $MnCl_2$, and the following buffers: succinate (○), glycerophosphate (▲), bis-TRIS-propane (□), HEPES (+) and TRIS (●)).

between the manganese concentration and the conversion rate is shown in **Figure 1**. The optimal manganese concentration (>10 mM) was much higher than expected for a catalyst. This might suggest that Mn^{2+} acts as reactant instead of catalyst, or only a fraction of the manganese dissolved is present in the catalytic active form.

The correlation between pH and conversion rate was determined using several buffers for pH values that are relevant for fermented dairy products (pH 4−8) (**Figure 2**). The pH of Gouda cheese is about 5.5 (*23*), and the intracellular pH of the microorganisms in cheese is close to neutral (*24*, *25*). A local maximum conversion was found at pH 5.5, but at pH 8 the conversion rate was even higher. At pH > 8, even higher conversion rates were expected, but the formation of a brown precipitate (MnO_2) at this pH resulted in noninterpretable results.

Varying the substrate concentration between 0.5 and 50 mM resulted in an almost linear increase of the conversion rate under the conditions tested (excess of oxygen is present in the air present in the headspace, 10 mM $MnCl_2$, pH 6.5) (data not shown). Oxygen is essential for the reaction because very low oxygen concentrations (0.1%) in the reaction vial led to a major decrease in the conversion rate (>90%). Moreover, oxygen was found to be consumed during the reaction (**Figure 3**).

For a better understanding of the reaction mechanism, it is important to know which products are formed. For the conversion of KICA to 2-methylpropanal, two carbon atoms have to be cleaved off, and the expected products are oxalic acid and/or carbon oxides (CO and CO_2), based on the homology with other reactions (*20*−*22*). The formation of these products was measured for reactions proceeding at pH 5.5 and 7.5 by use of direct inlet mass spectrometry, GC, and HPLC simultaneously. The HPLC method was also optimized for detection of other di- and mono-carbonic acids, in case the two carbon atoms would split off in a different manner. Oxalate and carbon dioxide were the only products detected, but the GC columns were not suitable for measurement of CO. These two products are formed via different mechanisms. Per molecule of 2-methylpropanal formed, either one molecule of oxalate or one molecule of CO_2 were formed. In the case where CO_2 was formed, this must have been accompanied by CO or formiate to complete the balance of elements. Formiate was not detected by HPLC analysis, which is in agreement with the findings of the conversion of *p*-hydroxyphenylpyruvate and phenylpyruvate (*21*, *22*, *26*). The quantitative headspace data were processed, taking gas−liquid equilibria (Henry's law ($K_{HCO_2} = 0.034$ M/atm, $K_{HO_2} = 0.0013$ M/atm (*27*)) and acid−base equilibria (to calculate $[HCO_3^-]$ ($K_{aHCO_3^-} = 2.3 \cdot 10^{-8}$ M)) into account. The results are combined in **Figure 3**. The increase of 2-methylpropanal

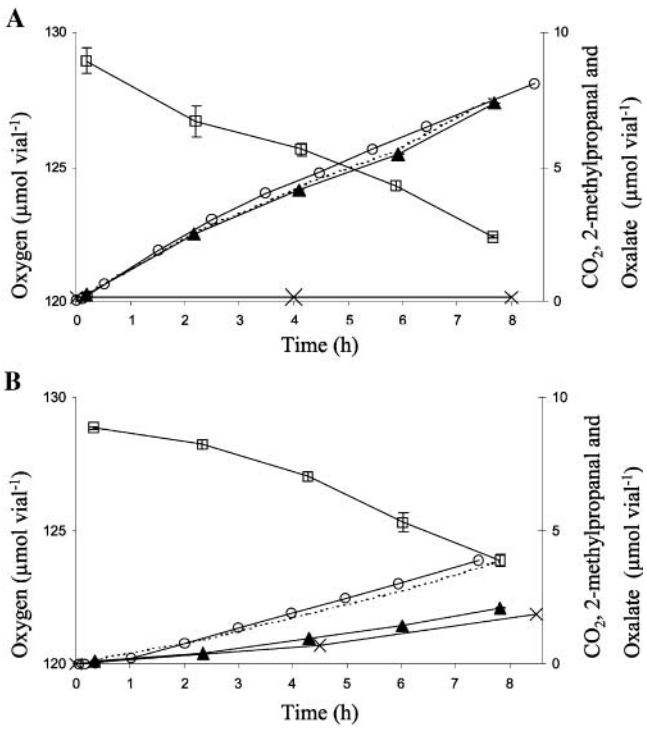


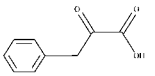
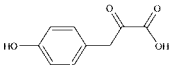
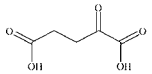
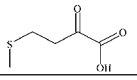
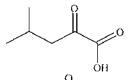
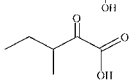
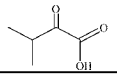
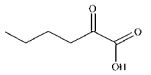
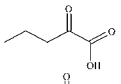
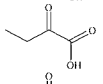
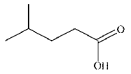
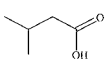
Figure 3. Oxygen (□) consumption and production of 2-methylpropanal (○), carbon dioxide (CO_2) (▲) and oxalate (×) during the chemical conversion of KICA at pH 5.5 (**A**) and 7.5 (**B**) in the presence of 10 mM manganese. The dotted line represents the sum of oxalate and CO_2.

and the increase of the sum of oxalate and CO_2 clearly correlate with ratios close to 1. However, the oxygen consumption in relation to the 2-methylpropanal production is slightly larger at high pH. This could again be caused by the oxidation of manganese to MnO_2, as described earlier. No further quantitative measurements were done to confirm this.

Our finding of chemical conversion of KICA shows that the chemical conversion of α-keto acids in fermented foods will not be limited to PPA and KMBA. More information on the conversion of several other substrates is therefore desired to estimate the impact of this reaction on the degradation of other α-keto acids in fermented products. Therefore, we incubated a variety of different α-keto acids and organic acids and determined their conversion by HPLC. The substrates were chosen based on their involvement in the amino acid catabolism or just as model substrates (linear keto acids and branched organic acids). In **Table 1**, the relative conversion rates and structures of the substrates tested are shown. The results indicate that the reaction is limited to α-keto acids, and in general, substrates with electron withdrawing side chains are converted faster. Also, two hydrogen atoms on the *β*-carbon atom seem to be preferable to one.

The reactivity of α-keto acids is mainly due to the existence of their enol tautomers (*17*, *18*). This was tested by measuring the conversion of the organic acid similar to KICA but lacking the keto-group (isohexanoic acid). This organic acid and other organic acids were not converted under the conditions tested. However, "linearized" KICA (2-keto hexanoic acid) was converted with approximately the same conversion rate as KICA. The organic acids tested are probably not able to form the reactive enol tautomers. Therefore, we conclude that the keto group (and the formation of the enol tautomer) is most likely essential for this oxidation reaction. The amount of the enol present in the equilibrium can be stabilized by intramo-

Table 1. Relative Conversion Rates of Several (α-Keto) Acids under Standard Conditions (Succinate, pH 5.5, 10 mM Mn)[a]

Substrate	Structure	Relative Conversion rate *	Related Amino acid
Phenylpyruvic acid (PAA)		100%	Phenylalanine
p -hydroxyphenylpyruvate		54%	Tyrosine
2-oxopentanedioic acid		32%	Glutamic acid
α-Ketomethylthio butyric acid (KMBA)		18%	Methionine
α-Ketoisocaproic acid (KICA)		29%	Leucine
α-Keto-ß-methyl-pentanoic acid		2%	Isoleucine
α-Ketoisovaleric acid		3%	Valine
α-Ketohexanoic acid		44%	
α-Ketopentanoic acid		47%	
α-Ketobutanoic acid		35%	
α-Ketopropanoic acid		5%	
Isohexanoic acid		0%	
Isopentanoic acid		0%	
Isobutanoic acid		0%	

[a] The standard deviation of the duplicate measurements is relative to the conversion of PAA. The amino acid substrates for the α-keto acids are mentioned in the last column. *Relative standard deviations of the values presented are in all cases ≤ 3.5%.

lecular hydrogen bonds and by conjugation of the carbon−carbon double bond with the carbonyl group (*17*, *28*). Keto−enol tautomerization is generally catalyzed by acid or base, but bivalent metal ions can also accelerate this reaction (*17*, *26*, *29*). A minimum in the conversion rates of KICA to 2-methylpropanal near neutral pH was confirmed in our experiments. We found a major catalytic effect of manganese ions; however, we did not find effects of other divalent metal ions, as was described for PPA conversion (*30*). If the main catalytic effect of manganese under the conditions tested is enhancement of the tautomerism by, for example, increasing the conversion rate or stabilizing the enol tautomer, keto−enol tautomery most probably determines the rate of the overall conversion. This is also indicated by the slow conversion of 2-oxo-3-methyl pentanoic acid and α-ketoisovaleric acid compared to their linear equivalent. The *β*-carbon atom of 2-oxo-3-methyl pentanoic acid and α-ketoisovaleric acid is methylated, leaving only one *β*-hydrogen atom, which is essential for the tautomerism, and thereby might slow the conversion.

On the basis of homology with several other ketones, the next step in the oxidation is most probably the formation of a peroxide on the *β*-carbon atom (*20*). This oxidation might proceed directly or via a radical mechanism (*20*, *21*). In the latter case, electron transfer of the nucleophilic enolate ion to molecular oxygen results in an α-keto radical (*28*, *29*, *31*). The propagation step involves the addition of molecular oxygen, resulting in a hydroperoxy radical (*20*). This radical can transfer the electron to another enol, leaving a new α-keto radical and a α-keto hydroperoxide (*29*, *31*).

The very reactive peroxide intermediary can react with the acid or keto carbon atom of the molecule resulting in a dioxylactone or dioxetanol. The cyclization to the dioxethanol is not a spontaneous reaction and only occurs under basic, non nucleophilic conditions (*22*). The unstable dioxylactone will decompose into an aldehyde (RC═O), and the carbon oxides (CO_2 and CO) (*22*). A hydrophilic solvent like water is also able to react with the keto function of the peroxide intermediary, resulting in a noncyclic intermediary (*22*). Oxalic acid and water

can be split off, leaving an aldehyde (RC═O). We identified both CO_2 and oxalate during conversion of KICA. This indicates that the conversion of KICA most probably proceeds via both pathways described for a nucleophilic environment. We also found that the sum of oxalate and carbondioxide correlated with the 2-methylpropanal production with ratios close to 1, and that the pathway via the noncyclic intermediary is enhanced at higher pH.

All α-keto acids, including the α-keto acids corresponding to amino acids, were converted under the mild conditions used (**Table 1**). This implies that the products of the other amino acid derived α-keto acids are also expected to be found in fermented products. In cheese and/or in the starter bacteria, not all parameters are as optimal as those under the conditions tested. The pH, for instance, varies during the fermentation and differs between the inside and outside of the bacterial cell. During growth, the pH of lactic acid bacteria is neutral, but after growth they are sometimes not able to maintain this pH, or will even lyse, which results in a decrease in the pH (*24*, *25*). The pH of Gouda cheese, for example, decreases from 7 to below 5.4 in the first 6 h of cheese production, after which it stays more or less stable (*23*). This pH is optimal for the chemical reaction to proceed. In smear-ripened and mould cheeses, the pH might increase to 7.5, due to the metabolic activities such as deamination (*32*). Some lactic acid bacteria, like *Lactobacillus casei* subsp. *casei* and *Lactobacillus plantarum* are able to accumulate manganese, leading to high local intracellular manganese concentrations (up to 50 mM) (*16*, *33*), which is also favorable for the chemical reaction. Particularly when transamination is stimulated, α-keto acids accumulate in the cheese matrix (up to 17.9% of the initial leucine concentration (*34*)), resulting in reasonable amounts of substrates present for the chemical reaction to proceed. However, the low oxygen concentration in cheese is very unfavorable, but some 2-methylpropanal was still formed at low oxygen concentrations (0.1% in headspace, which equals 1.3 μM in the reaction mixture), and depending on the type of cheese, particularly at the outside, some oxygen is available up to several weeks (*35*). The reaction will most probably proceed much more slowly under cheese ripening conditions than under the conditions used in this study, although under simulated Cheddar cheese conditions, spontaneous degradation of hydroxyphenylpyruvate to hydroxybenzaldehyde occurs (*36*), and both benzaldehyde and hydroxybenzaldehyde were found in semihard cheeses (*34*). Cheese ripening is a long process, which may take up to a year, and therefore we expect that the conversion rate will still be sufficient to change cheese flavor characteristics. In future work, this will be tested in cheese model systems, pilot cheese productions using selected starter cultures containing manganese accumulating strains, and transaminase overexpression mutants in combination with the addition of α-ketoglutarate to increase α-keto acid formation. A large difference in reaction rates between the substrates exist, the availability of the substrates in a product is very different, and the flavor characteristics of the aldehydes differ largely. More research has to be done on this topic. The chemical reaction described might be used as a new control point for aroma formation and flavor diversification in several fermented food products, not only by increasing flavor formation but also for preventing off-flavors. Increasing the oxygen concentration by using more permeable coatings, or selecting manganese accumulating strains might be relevant control parameters in this respect.

ACKNOWLEDGMENT

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LITERATURE CITED

(1) Engels, W. J. M.; Dekker, R.; de Jong, C.; Neeter, R.; Visser, S. A comparative study of volatile compounds in the water-soluble fraction of various types of ripened cheese. *Int. Dairy J.* **1997**, *7*, 255−263.

(2) Rychlik, M.; Bosset, J. O. Flavour and off-flavour compounds of Swiss Gruyere cheese. Identification of key odorants by quantitative instrumental and sensory studies. *Int. Dairy J.* **2001**, *11*, 903−910.

(3) Preininger, M.; Grosch, W. Evaluation of key odorants of the neutral volatiles of Emmentaler cheese by the calculation of odour activity values. *Lebensm.-Wiss. Technol.* **1994**, *27*, 237−244.

(4) Banks, J. M.; Brechany, E. Y.; Christie, W. W.; Hunter, E. A.; Muir, D. D. Volatile components in steam distillates of cheddar cheese as indicator indices of cheese maturity, flavour and odour. *Food Res. Int.* **1992**, *25*, 365−373.

(5) Smit, G.; Verheul, A.; van Kranenburg, R.; Ayad, E.; Siezen, R.; Engels, W. J. M. Cheese flavour development by enzymatic conversions of peptides and amino acids. *Food Res. Int.* **2000**, *33*, 153−160.

(6) McSweeney, P. L. H.; Sousa, M. J. Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Lait* **2000**, *80*, 293−324.

(7) Yvon, M.; Rijnen, L. Cheese flavour formation by amino acid catabolism. *Int. Dairy J.* **2001**, *11*, 185−201.

(8) Weimer, B.; Seefeldt, K.; Dias, B. Sulfur metabolism in bacteria associated with cheese. *Ant. Leeuwenhoek* **1999**, *76*, 247−261.

(9) Williams, A. G.; Noble, J.; Tammam, J.; Lloyd, D.; Banks, J. M. Factors affecting the activity of enzymes involved in peptide and amino acid catabolism in nonstarter lactic acid bacteria isolated from Cheddar cheese. *Int. Dairy J.* **2002**, *12*, 841−852.

(10) Visser, F. M. W. Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. II. Development of bitterness and cheese flavour. *Neth. Milk Dairy J.* **1977**, *31*, 188−209.

(11) Engels, W. J. M. Volatile and nonvolatile compounds in ripened cheese: their formation and their contribution to flavour. Ph.D. Thesis, Wageningen Agricultural University, 1997.

(12) Gao, S.; Oh, D. H.; Broadbent, J. R.; Johnson, M. E.; Weimer, B. C.; Steele, J. L. Aromatic amino acid catabolism by lactococci. *Lait* **1997**, *77*, 371−381.

(13) Smit, B. A.; Engels, W. J. M.; Wouters, J. T. M.; Smit, G. Diversity of L-leucine catabolism in various microorganisms involved in dairy fermentations and identification of the rate controlling step in 3-methylbutanal formation. *Appl. Microbiol. Biotechnol.* **2003**, *Online First*, DOI: 10.1007/s00253-003-1447-8.

(14) Smit, B. A.; Engels, W. J. M.; Bruinsma, J.; Van Hylckama Vlieg, J. E. T.; Wouters, J. T. M.; Smit, G. Development of a High Throughput-screening method to test flavour-forming capability of food microorganisms, in Press.

(15) Villablanca, M.; Cilento, G. Oxidation of phenylpyruvic acid. *Biochim. Biophys. Acta* **1987**, *926*, 224−230.

(16) Nierop Groot, M. N.; de Bont, J. A. M. Involvement of manganese in conversion of phenylalanine to benzaldehyde by lactic acid bacteria. *Appl. Environ. Microbiol.* **1999**, *65*, 5590−5593.

(17) Carey, F. A.; Sundberg, R. J. 7.3 Enols and enamines. In *Advanced organic chemistry*; F. A. Carey and R. J. Sundberg, Eds.; Plenum Press: London, 1990; pp 416−421.

(18) Pitt, B. M. Oxidation of phenylpyruvates to aromatic aldehydes and oxalate. *Nature* **1962**, *196*, 272−273.

(19) Doy, C. H. Alkaline conversion of 4-hydroxyphenylpyruvic acid to 4-hydroxybenzaldehyde. *Nature* **1960**, *14*, 1529−1531.

(20) Carey, F. A.; Sundberg, R. J. 12 Oxidations. In *Advanced organic chemistry*; F. A. Carey and R. J. Sundberg, Eds.; Plenum Press: London, 1990; pp 651−657.

(21) Gersmann, H. R.; Bickel, A. F. Autoxidation of ketones and esters in basic solution. *J. Chem. Soc. B* **1971**, 2230−2237.

(22) Jefford, C. W.; Knöpfel, W.; Cadby, P. A. Oxygenation of methyl p-methoxyphenylpyruvate. alpha-keto-β-peroxylactone and dioxetanol intermediates. *Tetrahedron Lett.* **1978**, *38*, 3585−3588.
(23) Walstra, P.; Noomen, A.; Geurts, T. J. Dutch type varieties. In *Cheese*: *Chemistry*, *physics*, *and microbiology*; P. FOX, Ed.; Chapman & Hall: London, 1993; pp 39−82.
(24) Molenaar, D.; Abee, T.; Konings, W. N. Continuous measurement of the cytoplasmic pH in *Lactococcus lactis* with a fluorescent pH indicator. *Biochim. Biophys. Acta* **1991**, *1115*, 75−84.
(25) Hugenholtz, J.; Starrenburg, M. J. C.; Abee, T. Control of internal pH in lactic acid bacteria. In *Proceedings*: *Lactic acid bacteria*; Adria Normandie: Caen, France, 1997; pp 469−470.
(26) Büchner, T.; Kirberger, E. Über die enol-keto-tautomerie der *p*-oxy-phenylbrenztraubensaure. *Biochim. Biophys. Acta* **1952**, *8*, 401−406.
(27) Sander, R. Compilation of Henry's law constants for inorganic and organic species of potential importance in environmental chemistry. *www..mpch-mainz.mpg.de*/∼sander/res/henry.html. 1999.
(28) Russel, G. A.; Bemix, A. G. The oxidation of carbanions. I. Oxidation of triaryl carbanions and other tertiairy carbanions. *J. Am. Chem. Soc.* **1966**, *88*, 5491−5497.
(29) Bailey, E. J.; Barton, D. H. R.; Elks, J.; Templeton, J. F. Compounds related to the steroid hormones. part IX. Oxygenation of steroid ketones in strongly basic medium: A new method of preparation of 17a-hydroxypregnan-20-ones. *J. Chem. Soc.* **1962**, 1578−1591.
(30) Nierop Groot, M. N.; De Bont, J. A. M. Conversion of phenylalanine to benzaldehyde initiated by an aminotransferase in *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **1998**, *64*, 3009−3013.
(31) House, H. O. *Modern Synthetic Reactions*, 2nd ed.; Benjamin-Cummings: Menlo Park, CA, 1972.
(32) Bockelmann, W. The production of smear cheeses. In *Dairy Processing*: *Improving quality*; G. Smit, Ed.; Woodhead Publishing Limited: Cambridge, 2003; pp 470−491.
(33) Archibald, F. Manganese: Its aquisition by and function in lactic acid bacteria. *Crit. Rev. Microbiol.* **1986**, *13*, 63−109.
(34) Yvon, M.; Berthelot, S.; Gripon, J. C. Adding alpha-ketoglutarate to semi-hard cheese curd highly enhances the conversion of amino acids to aroma compounds. *Int. Dairy J.* **1998**, *8*, 889−898.
(35) Van den Tempel, T.; Gundersen, J. K.; Nielsen, M. S. The microdistribution of oxygen in Danablu cheese measured by a microsensor during ripening. *Int. J. Food Microbiol.* **2002**, *75*, 157−161.
(36) Gao, S.; Mooberry, E. S.; Steele, J. L. Use of 13C nuclear magnetic resonance and gas chromatography to examine methionine catabolism by lactococci. *Appl. Environ. Microbiol.* **1998**, *64*, 4670−4675.

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