Volatile and non-volatile compounds in ripened cheese: their formation and their contribution to flavour

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Volatile and non-volatile compounds in ripened cheese: their formation and their contribution to flavour

PROEFSCHRIFT

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STELLINGEN

1. De door Bosset en Gauch gerapporteerde kwantitatieve gegevens betreffende vluchtige verbindingen in diverse kaassoorten dienen met de nodige voorzichtigheid geïnterpreteerd te worden. Er mag bij massaspectrometrische bepalingen namelijk niet van uitgegaan worden dat iedere verbinding in dezelfde mate fragmenteerbaar is.

   Bosset, J.O. and R. Gauch (1993) Comparison of the volatile flavour compounds of six European "AOC" cheeses by using a new dynamic headspace GC-MS method. Int. Dairy J. **3**:359-377.

2. De tijdens de kaasrijping gevormde peptiden en aminozuren dragen alleen indirect bij aan de specifieke kaassmaak.

   Dit proefschrift.

3. Onderzoek naar de vorming van vluchtige zwavelverbindingen maakt je niet altijd geliefd bij je collega's.

4. Het gegeven dat we dankzij ons "geurgeheugen" een vaak levenslange aversie krijgen tegen bepaalde voedingsmiddelen maakt van het houden van geur- en smaakkeuringen een bedenkelijke bezigheid.

5. De conclusie door Yvon *et al.* dat het door hen gezuiverde aminotransferase ongetwijfeld betrokken is bij de biosynthese van phenylalanine en tyrosine is voorbarig.

   Yvon, M., S. Thirouin, L. Rijnen, D. Fromentier and J.C. Gripon. (1997) An aminotransferase from *Lactococcus lactis* initiates conversion of amino acids to cheese flavor compounds. Appl. Environ. Microbiol. **63**:414-419.

6. Het bestaan van in publicaties veelvuldig genoemde "umamipeptiden", met een hartige, MSG- of bouillon-achtige smaak, is hoogst onwaarschijnlijk.

   Van den Oord, A. and P.D. van Wassenaar (1997) Umami peptides: assessment of their alleged taste properties. Z. Lebensm. Unters. Forsch. A **205**:125-130.

7. De merknaam van tekst- en dataverwerkende software suggereert vaak meer gebruiksgemak dan in de praktijk wordt ervaren.

8. Hoewel vrijwel alle steden in Nederland zich afficheren in termen van uniek en karakteristiek, zijn de winkelgebieden in diezelfde steden toch vooral identiek.

9. De term WYGIWYS (What You Get Is What You See) benadert de werkelijkheid soms beter dan de gebruikelijke term WYSIWYG (What You See Is What You Get).

10. Ook een melkzuurbacterie heeft wel eens last van stress.

    Rallu, F., A. Gruss and E. Maguin (1996) *Lactococcus lactis* and stress. Ant. Leeuwenhoek **70**:243-251.

Stellingen behorende bij het proefschrift:
Volatile and non-volatile compounds in ripened cheese: their formation and their contribution to flavour

Wim Engels, Wageningen, 16 december 1997

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Chapter 1

General introduction

General introduction

A variety of lactic acid bacteria and other microrganisms are used in the production of a wide range of fermented dairy products. The main purpose of their use is to ensure a proper preservation of the fermented product. The rapid conversion of lactose, present in milk, into lactic acid is the most important feature of lactic acid bacteria in this respect. The resulting reduction of pH, a concomitant lowering of the redox potential and the absence of lactose inhibits growth of undesired bacteria in the dairy product, e.g. cheese.

In addition to the production of lactic acid, organisms used in fermented dairy products also determine flavour and texture of the product. Because of the growing demand for tasty and healthy food, the development of new or improved fermented products is considerably stimulated. This requires a thorough understanding of the processes involved in the formation of flavour compounds. This thesis presents investigations on flavour formation in cheese.

1 Cheese

Cheese is one of the most important fermented dairy products. Its manufacture is essentially a process in which milk protein (casein) and fat are separated from the rest of the milk [32]. This is achieved by coagulation of the caseins, which is initiated by the specific action of chymosin in rennet on κ-casein. The resulting para-κ-casein coagulates into a casein gel which entraps the fat and bacteria present in the milk, as well as part of the rennet. The gel is cut or stirred to promote syneresis (extrusion of the whey). The syneresis is further promoted by heating the curd/whey mixture (cooking). In Gouda cheese production this is done by addition of hot water [107].

In the next steps of cheese production, whey is removed by draining and pressing, resulting in fresh cheese with a closed rind. During the pressing phase, and the subsequent holding stage in the cheese vats, the fermentation of residual lactose by lactic acid bacteria is responsible for an important lowering of the pH. The cheese is salted, e.g. by keeping it in a brine (Gouda cheese) or by adding salt directly to the milled curd (Cheddar cheese). During brining lactose conversion continues until all the lactose has been fermented, whereby the pH of the cheese is lowered to about 5.2-5.4 [107]. The conversion of lactose in Gouda usually takes about 24 hours; however, due to the direct addition of salt, in Cheddar cheese it may take more than a week before lactose fermentation is complete. In addition to lactose fermentation, oxygen present is metabolized rapidly and a low redox potential is created [32, 36, 107].

The next and most time-consuming stage in the production of most cheese types is ripening, which for Gouda cheese takes place at 12-15 °C. The ripening process will be discussed in more detail in the next sections of this chapter.

Differences between cheese varieties are partially determined by variations in the procedures utilised during the early stages of cheese manufacture, e.g. curd syneresis, cooking and salting [48, 79]. A major role is played by the starter, and possibly non-starter, lactic acid bacteria and other microorganisms present during cheese production and ripening. The lactic acid starter cultures used by the Dutch dairy industry, producing mainly Gouda, Edam and Maasdam-type cheese, primarily consist of strains of mesophilic *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* [107]. Starter cultures often also contain bacteria capable of utilising the citrate present in milk, e.g. members of the genus *Leuconostoc* and *Lactococcus lactis* subsp. *lactis* var. *diacetylactis*. One of the conversion products of citrate is carbon dioxide which is important for the formation of eyes in cheese [20]. In Maasdam-type cheeses propionic acid bacteria are added for additional carbon dioxide production and formation of large eyes.

Other types of cheese develop a surface flora of moulds and yeasts, e.g. Brie and Camembert [60]. Blue vein cheeses contain moulds within the cheese matrix e.g. *Penicillium roqueforti* [60].

2 Cheese ripening

Cheese ripening is a complex process which primarily involves glycolysis, lipolysis and proteolysis [35] (Figure 1).

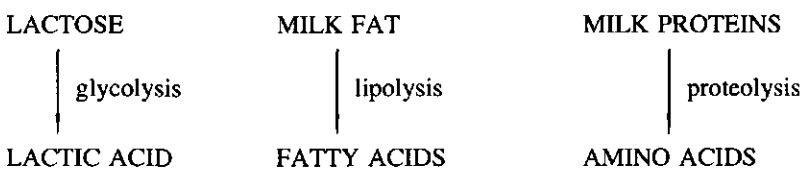

Figure 1. Major conversion processes during cheese ripening.

The conversion of lactose into lactate provides the lactic acid bacteria with energy during growth in milk. The conversion of lactose can either be homofermentative or heterofermentative. During homofermentative lactose degradation only lactate is produced, whereas heterofermentative degradation yields lactate, acetate, carbon dioxide and ethanol. Some lactic acid bacteria, such as *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* and *Leuconostoc* subsp., are also able to utilise citrate. The conversion of citrate, a normal component of milk, by some lactic acid bacteria yields carbon dioxide and carbonyl compounds, such as diacetyl [24].

The fat fraction in cheese is important for the perception and development of flavour [34]. The first step in the decomposition of fat is lipolysis which causes the formation of free fatty acids [89] which also may act as precursors for other flavour compounds

[37, 108]. Although only limited lipolysis occurs in most cheese varieties, fatty acids and products formed thereof are important components in the flavour of some cheese types [35]. Examples are Blue cheeses [39], Camembert cheese [73] and Italian varieties [16].

The most important biochemical process for flavour and for texture formation in hard-type and semi-hard-type cheeses undoubtedly is proteolysis [99]. The extent of proteolysis ranges from limited (e.g. Mozzarella) to very extensive (e.g. Blue cheeses and mould-ripened cheeses) [34]. The process of proteolysis in ripening cheese has been investigated thoroughly and will be discussed in some detail in the next section.

Products of proteolysis, i.e. peptides and amino acids, together with other non-volatile compounds are found in the water-soluble fraction (WSF) of ripened cheese [3, 58, 99]. The WSF is considered to make the greatest contribution to cheese flavour [69] and this fraction has been investigated extensively. Besides non-volatiles, it also contains numerous volatile compounds, originating from breakdown of amino acids, fat, lactose and citrate during ripening [11, 16, 44, 47, 90].

Cheddar cheese has been most comprehensively studied, and various methods have been described to obtain a WSF from this type of cheese [69, 57]. Many studies on the fractionation of WSF obtained from several types of cheese, e.g. by ultrafiltration [3], gel filtration [58] and HPLC [80], and methods applied for extraction of volatiles from WSF [7, 11, 37, 72, 109, 111] have been published. The reader is referred to the original articles for detailed information.

In Table 1, typical examples of volatiles found in various types of ripened cheese are shown. As can be seen the volatiles belong to various groups of organic compounds, e.g. fatty acids, esters, aldehydes, etc. The flavour attributes of the volatile compounds can range from pleasant-fruity for esters to putrid-unclean for sulphur compounds.

Table 1. Major groups of volatiles formed during cheese ripening.

Compounds	Typical examples	Especially found in (Cheese type)	Reference
fatty acids	acetic acid	Gruyère, Parmesan, Camembert	8, 73, this thesis
	propionic acid	Emmental, Maasdam	93, this thesis
	butyric acid	Gruyère, Parmesan, Camembert	8, 12, 73
esters	ethyl butanoate	Gruyère, Parmesan	8, this thesis
	ethyl decanoate	Roquefort	37
aldehydes	3-methyl-butanal	Proosdij, Parmesan	8, this thesis
	2-methyl-butanal	Parmesan	8
	benzaldehyde	Comté	11
alcohols	1-butanol	Gruyère, Parmesan, Maasdam	8, this thesis
	3-methyl-1-butanol	Edam, Maasdam, Parmesan	8, this thesis
	phenylethanol	Camembert	73
ketones	2-heptanone	Roquefort, Camembert	37, 49, 73
	2-nonanone	Roquefort, Camembert	37, 49, 73
	2-butanone	Edam	this thesis

Compounds	Typical examples	Especially found in (Cheese type)	Reference
sulphur compounds	dimethyldisulphide	Limburger, Cheddar, Gouda	81, 96, this thesis
	methional	Cheddar, Emmental	18, 83
various components	phenol	Limburger	96
	limonene	Fontina	11
	δ-decalactone	Emmental	83
	4-hydroxy-2,5-dimethyl-3(2H)-furanone	Emmental	83

3 Proteolysis during cheese ripening

Of the three primary biochemical events (Figure 1) that occur during cheese ripening, proteolysis is the most complex and, according to many investigators [33, 79, 99], the most important one. The degradation of caseins plays a critical role in the development of texture and flavour of cheeses. The proteolytic enzymes involved in the ripening of cheese originate from various sources, e.g. milk, coagulant, starter bacteria and non-starter bacteria or other microorganisms. In Figure 2 the general pathway for the breakdown of caseins, and the enzymes involved, during the manufacture and ripening of cheese is shown.

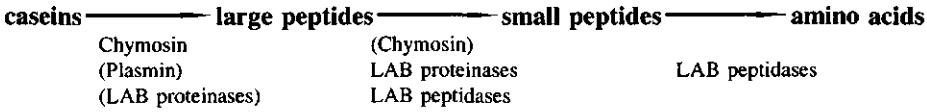

Figure 2. Breakdown of caseins to amino acids during cheese ripening. LAB = Lactic Acid Bacteria. The enzymes in parentheses are regarded to play a less important role.

3.1 Proteinases from milk and coagulant

Chymosin and pepsin, the main proteinases in traditional rennets used for cheese making, are aspartic proteinases from the stomach of calves. The principal role of the rennet enzymes is to coagulate milk by specifically hydrolyzing the micelle-stabilizing protein κ-casein at the Phe_{105}-Met_{106} bond [106]. This results in the appearance of para-κ-casein and caseinomacropeptide. The high preference of chymosin, and to a lesser extent also of pepsin, for splitting of κ-casein and their much lower activity towards other caseins, is an essential property of the rennet enzymes [99]. During the ripening residual rennet enzyme activity is reponsible for initial proteolysis of other caseins in cheese. In that stage the primary site of chymosin action is on the Phe_{23}-Phe_{24} bond of α_{s1}-casein [26]. Cleavage of this bond is believed to be responsible for softening of cheese texture [34, 78]. The peptide α_{s1}-CN(f1-23) is further hydrolysed by proteolytic enzymes from the starter organisms [27, 30] whereas α_{s1}-CN(f24-199) is degraded in

cheese to small peptides and amino acids by combined action of chymosin and proteolytic enzymes from the starter organisms [29, 31]. In solution, the hydrolysis of β-casein by chymosin occurs at several sites, as was shown by Visser and Slangen [100]. The most sensitive bond is Leu_{192}-Tyr_{193} and the resulting C-terminal 193-209 fragment of β-casein is an extremely bitter peptide [99]. In most cheeses the formation of this and other (bitter) peptides from β-casein is very slow due to the presence of salt [99], but it could still be significant during the initial phase of ripening of Gouda cheese [31]. Recent research by Exterkate et al. [31] showed that a combination of factors which are characteristic for the cheese environment, e.g. salt, Ca^{2+}, low pH, a restricted water content and possibly the cheese matrix itself, influence the cleavage of α_{s1}- and β-casein.

The indigenous milk proteinase, plasmin, a serine proteinase, also contributes to proteolysis in cheese, especially of high-cooked varieties, such as Emmental [35]. The higher cooking temperature results in (partial) inactivation of chymosin. The role of plasmin in proteolysis in Gouda cheese is, however, thought to be limited [99]. In solution, plasmin mainly cleaves β- and α_{s2}-casein [91], although α_{s1}-casein is cleaved as well [42, 70]. In cheese, β-casein hydrolysis by plasmin becomes apparent via the appearance of γ-casein after gel-electrophoresis [98].

In summary, the initial proteolysis in most cheeses by residual coagulant enzyme and to a lesser extent by plasmin, results in the formation of large and medium-sized peptides. These peptides are further degraded by proteolytic enzymes from starter bacteria and, in some cheese varieties, non-starter microorganisms.

3.2 Proteolytic enzymes from cheese microorganisms

3.2.1 Extracellular proteinases

Most lactic acid bacteria used in dairy systems, such as the genera *Lactococcus* and *Lactobacillus*, rely on a complex system of proteolytic enzymes for provision of amino acids from the growth medium, since these bacteria have only limited biosynthetic abilities [10, 17]. The degradation of caseins yields both peptides and amino acids, such as Ile, Leu, Val, His and Met that are essential for rapid growth of lactic acid bacteria in milk [17, 82, 94]. Although lactococci, the most widely used cheese-starter organisms, are only relatively weakly proteolytic, they may possess a proteinase and a wide range of peptidases which together are principally responsible for the formation of small peptides and amino acids in cheese [33, 99].

The proteinase in lactic acid bacteria is an extracellular cell-envelope-bound enzyme. Two main types of proteinase, P_I and P_{III}, have been initially recognized among lactococci, which differ by their specificity towards caseins [101]. The primary substrate of P_I-type enzymes is β-casein, although κ-casein is also degraded, while P_{III}-type enzymes degrade α_{s1}-, β- and κ-caseins [84, 101]. Apart from P_I-type (e.g. from *Lactococcus lactis* subsp. *cremoris* HP) or P_{III}-type (e.g. from *Lactococcus lactis* subsp. *cremoris* SK11) proteinase, several strains of *Lactococcus* contain mixed P_I- and P_{III}-

type proteinases [28, 101]. Based on the specificities of cell-envelope proteinases (CEPs) toward α_{s1}-CN(f1-23) the designations CEP_I, CEP_{II} and CEP_{III} were proposed, together with $CEP_{I/III}$ and $CEP_{II/III}$ for mixed-type proteinases [28].

The products resulting from the action of purified proteinases from *Lactococcus lactis* strains on α-, β- and κ-casein, in solution have been partially analyzed. β-Casein is mainly hydrolysed in the C-terminal region of the protein by P_I-type proteinase [74, 104]. More than hundred different oligopeptides, mainly consisting of 4-10 residues, were identified by Juillard et al. [54] after *in vitro* action of purified P_I-type proteinase of strain Wg2 on β-casein. Various peptides liberated from β-casein by the action of P_{III}-type proteinases have also been identified [102]. The *in vitro* hydrolysis of several bonds of κ-casein by P_I-type as well as P_{III}-type proteinases has been reported [75, 86, 103]. The degradation of α_{s1}-casein is mainly attributed to the P_{III}-type proteinases as was established during experiments with partially purified enzymes [101].

From several studies it has become clear that under cheese ripening conditions the breakdown of caseins by cell-envelope proteinases is different from the breakdown under *in vitro* conditions. Exterkate et al. [30] have revealed that, under conditions prevailing in cheese, various peptides are formed from the primary product α_{s1}-CN(f1-23) of chymosin action on α_{s1}-casein. Both purified P_I- and P_{III}-type proteinases were able to cleave α_{s1}-CN(f1-23) and several cleavage sites, e.g. bonds between residues 8-9, 9-10, 13-14, 16-17, 17-18 and 21-22, were identified. It appeared that under simulated cheese ripening conditions (pH 5.2, 4 % NaCl, w/v) deviations occur from the conversion of α_{s1}-CN(f1-23) observed in solution under conditions optimal for enzyme action [5, 30]. *In situ* (cell-bound) proteinase action largely reflected the specificity of the soluble enzymes, although the pH dependence of the *in situ* P_I-type proteinase clearly deviated from that of the purified enzyme [30]. The *in vitro* breakdown of α_{s1}-CN(f24-199) fragments, obtained by action of chymosin on α_{s1}-CN(f24-199), at pH 5.2 and 4 % NaCl (w/v) with P_I-type cell-envelope proteinase resulted in the formation of fragments with various chain lengths [29].

3.2.2 Intracellular peptidases

From several studies in the last decade (see for review Kunji et al. [59]) it is evident that during growth in milk a multitude of oligopeptides are produced from caseins by the cell-envelope-associated proteinase. In order to utilize the amino acids from these peptides for biosynthesis, these peptides have to be hydrolysed further by peptidases. Biochemical and genetic studies have indicated that probably all peptidases of *Lactococcus lactis* are located intracellularly [82]. Lactococci therefore possess several distinct transport systems for transport of peptides across the cell-membrane. A large fraction of the peptides formed is taken up by the lactococcal cells via the, ATP-dependent, oligopeptide transport system. Peptides up to lengths of 8 amino acids are transported through this system [59]. Mutants which lack a functional oligopeptide transport system are unable to grow on milk and, therefore, it seems absolutely necessary for lactococci in order to use milk peptides as nitrogen source [53, 95]. A

minor role in the uptake of essential amino acids in the form of peptides is played by the di-tripeptide transport systems (DtpT and DtpP) [82]. Several ATP as well as proton-motive-force driven amino acid transport systems, e.g. for Glu/Gln, Leu/Ile/Val, Ser/Thr and Met, were also characterized in lactococci but during growth in milk the uptake of free amino acids is very low. The di-tripeptide transport systems and the amino acid transport systems possibly play a role in maintaining the correct balance in amino acids and small peptides inside the cell [59].

For the intracellular degradation of actively transported peptides, a multitude of peptidases is present in the cytoplasm of the cells. The peptidases of lactococci were divided into two classes by Kunji et al. [59]:
(1) primary peptidases, that generate free amino acids from oligopeptides directly (e.g. PepN, PepA and PepI) and
(2) secondary peptidases, that require degradation of peptides by other lactococcal peptidases, prior to or after their action, to complete hydrolysis to the level of free amino acids (e.g. PepO, PepF, PepX and PepR).

The exact role of the various peptidases for growth in milk is not yet elucidated. However, their concerted action is essential for the complete hydrolysis of the numerous peptides from milk protein.

During cheese ripening, active uptake of peptides by lactococci is probably strongly inhibited because of a lack of lactose. However, despite their intracellular location lactococcal peptidases will play an important role in the formation of free amino acids under these conditions because of their release into the cheese matrix from lysed cells [22, 62, 71].

From the discussion in this section, it is clear that there is considerable knowledge of both proteolysis of caseins during growth of lactic acid bacteria in milk and of proteolysis during cheese ripening. It is evident that starter cells exist in other physiological states in ripening cheese compared with cells growing in milk. In cheese the relatively low pH (5.2-5.4) and high NaCl content (approximately 4 %, w/v, for Gouda cheese) will influence the specificity and stability of enzymes involved in ripening [23, 29, 30].

Besides *Lactococcus lactis* many other species of lactic acid bacteria, e.g. thermophilic *Lactobacillus* species, and other, non-starter, microorganisms may contribute to proteolysis in cheese [14, 34, 40, 41]. Adjunct organisms in the production of mould-ripened cheeses (*Penicillium* subsp.) and smear-ripened varieties (*Brevibacterium linens*) often have strong proteolytic activity compared with lactic acid bacteria, whereas *Propionibacterium* subsp. in Swiss varieties are considered to be less proteolytic [33, 55]. Various reports on proteolytic systems of these organisms have been published and for more detailed information the reader is referred to these reports and to references [33] and [34].

4 Cheese flavour from products of proteolysis

In the previous section of this chapter it was shown that in cheese the hydrolysis of caseins by proteolytic enzymes produces peptides and free amino acids. The amino acids formed play an important role in the development of cheese flavour [23]. However, the direct role of amino acids for cheese flavour is limited. The very heterogeneous nature of cheese peptides makes it difficult to correlate flavour with particular peptides. Large and medium-sized peptides are probably not major contributors to cheese flavour. Short-chain peptides can have important flavour characteristics [110], but often form (together with larger peptides) the basis of off-flavours in cheese, such as bitter. Bitter peptides have been isolated from Gouda [105] and Cheddar cheese [46, 65]. The precise relationship between peptide structure and bitterness is not known although bitter peptides tend to be rich in hydrophobic amino acids and often also in proline [65].

Previous research [3, 4, 88] has demonstrated that free amino acids and very small peptides may provide a brothy or other background flavour to cheese. In Table 2, the flavours of some individual amino acids and small peptides are listed.

Table 2. Taste of some amino acids and peptides.

Compound	Flavour	Reference
L-Gly, L-Ala, L-Pro	sweet	92
L-Val, L-Lys, L-Met	bitter and sweet	45, 92
L-Leu, L-Ile, L-Phe, L-Trp	bitter	45, 92
L-Asp	acid	45, 92
L-Glu	broth	92
Glu-Glu, Glu-Asp, Glu-Ser	broth	19
Glu-Val-Leu-Asn	bitter	87
Val-Pro-Pro-Phe-Leu-Gln	bitter	105

Catabolism of amino acids during cheese ripening as a source of flavour compounds has been suggested by many researchers [23, 47, 61, 67, 96, 97]. The amino acids are exposed to enzymic but probably also to chemical reactions. Microorganisms present in the cheese are the source of the enzymes but they also could favour the conditions for chemical reactions, e.g. by lowering the redox potential [36, 68, 79]. A number of compounds, including ammonia, amines, aldehydes, alcohols, phenols and sulphur compounds can result from catabolism of amino acids. In Table 3, examples of amino acid-derived cheese volatiles are given.

Table 3. Examples of products formed by breakdown of amino acids during cheese ripening.

Amino acid	Volatile product	Flavour	Reference
Leu	3-methyl-1-butanol	fresh cheese, fruity	72, 73
Ile	2-methylbutanal	malty, harsh	25, 38, 76
Met	methanethiol	onion, cheese	38, 81
Phe	phenylacetaldehyde	rose	38
Tyr	phenol	phenol, medicinal	25, 72
Thr	acetaldehyde	"green", yoghurt	6
Val	2-methylpropanal	malty, harsh	25, 38, 76

In mould- and smear-ripened cheese varieties the role of these products of amino acid breakdown is particularly significant [34, 60]. For example, ammonia contributes to the aroma of a number of cheeses, such as Camembert, Gruyère and Comté, and results from amino acid deamination [47]. Non-starter microorganisms such as coryneform bacteria (e.g. *Brevibacterium linens*) and moulds (e.g. *Penicillium camemberti* and *Penicillium roqueforti*) are capable of producing relatively large amounts of ammonia and other volatiles from amino acids [13, 50, 64, 85]. The degradation of amino acids not only takes place via the action of deaminases but also by enzymes such as decarboxylases, transaminases and enzymes converting amino acid side-chains [16, 47]. Some general pathways of amino acid catabolism are shown in Figure 3.

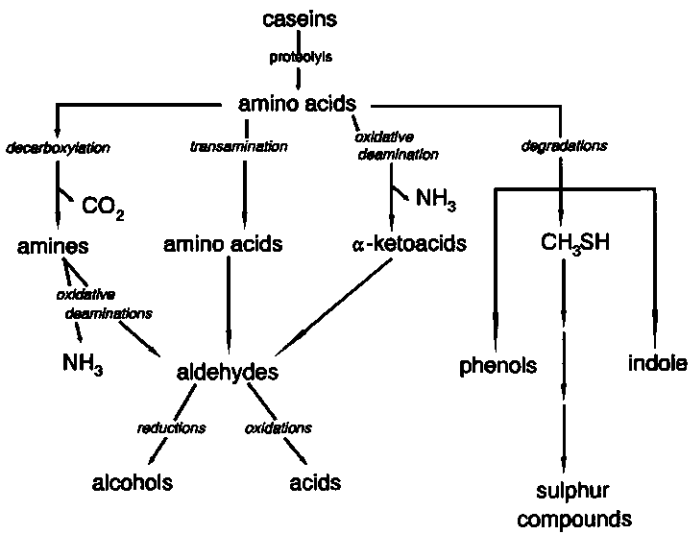

Figure 3. Pathways of breakdown of amino acids. Adapted from Hemme et al. [47].

The process of decarboxylation of some amino acids leading to the formation of biogenic amines, probably by enzymes from contaminating microflora in cheese, has been reported by Joosten et al. [51, 52]. However, no evidence has been found that decarboxylating enzymes from mesophilic lactococci used for the production of hard-type cheeses, such as Gouda and Cheddar, were involved in this process [52, 89]. The occurrence of oxidative deamination and transamination has been demonstrated in cheese curd [77, 89], although it was unclear whether enzymes from the starter organisms were involved in the reactions. One deamination pathway which has been fairly well studied in starters is the arginine deiminase pathway found in *Lactococcus lactis* subsp. *lactis* [21]. The transaminative degradation of phenylalanine by *Brevibacterium linens* and yeast from Camembert cheese was reported by Lee and Richard [63]. In Cheddar cheese, the catabolism of phenylalanine and other aromatic amino acids by mesophilic lactobacilli is also believed to play a significant role in the development of undesirable flavours [43].

The relevance of decomposition products of sulphur-containing amino acids, especially methionine, has been known for years [1, 47, 68, 97] and their presence is reported in various cheese types [8, 37, 81, 97]. The formation of volatile sulphur compounds, e.g. H_2S and methanethiol, is usually attributed to the action of enzymes from non-starter organisms e.g. *Brevibacterium linens* and *Pseudomonas* species [66]. Various low molecular-weight sulphur compounds occur regularly in headspace samples of Cheddar cheese [96]. Non-enzymic pathways for the formation of volatile sulphur compounds have first been suggested by Manning [68].

The formation of cheese flavours by reactions between dicarbonyls and amino acids has been suggested by Kowalewska et al. [56]. Later Griffith and Hammond [38] could generate flavour notes like "rose-like" and "cheesy" from mixtures of amino acids (e.g. valine, leucine, methionine and phenylalanine) and dicarbonyls. The presence of dicarbonyls, such as glyoxal, methylglyoxal and acetoin, in various types of cheese [9] further supported the possible role of these, non-enzymically (via Strecker degradation) produced, amino acid-carbonyl complexes.

Amino acid degradation by mesophilic starter lactococci generally is regarded to be less intense in semi-hard-type cheeses, such as Gouda and Cheddar, than in varieties in which moulds or non-lactic acid bacteria are present [2]. However, it can be assumed that the role of mesophilic starter lactococci during cheese ripening is not limited to the production of substrates, such as amino acids, and to supplying the conditions, e.g. a low redox potential [68], which favour the conversion of these substrates. During the course of the research described in this thesis the in-vitro catabolism of methionine and other amino acids, by enzymes from mesophilic lactococci, has been reported [15, 112]. Proving that these reactions also occur in cheese is a more difficult problem. In cheese the conditions for amino acid breakdown are less favourable due to the low pH, high salt concentration and the low temperature of ripening. Lysis of cells during cheese ripening will lead to release of the intracellular enzymes into the cheese matrix. This

can result in enzymic pathways becoming incomplete or out of balance and thus resulting in endproducts different from those produced by intact bacteria [23].

5 Outline of the present work

The formation of flavour components is an essential aspect of the cheese ripening process. The conversion of lactose and the degradation of caseins and fats carried out by lactic acid bacteria and other microorganisms during cheese production and ripening are important processes in this respect. Although numerous studies about cheese-flavour compounds have been published, still relatively little is known about the key flavour components of many types of cheese. In certain cheese types, e.g. those ripened with surface flora or with added lipolytic enzymes, compounds dominating the flavour can be designated [73, 108]. However, in ripened cheeses produced mainly with mesophilic lactococci, such as Cheddar and Gouda, this is a more difficult task. Identification of important flavour compounds as well as unravelling their route of formation are profound challenges.

The aim of the present work was (a) to determine the contribution to flavour of various compounds present in the water-soluble fraction of cheese and (b) to investigate the formation of flavour components from amino acids by lactococcal enzymes. In this chapter a brief overview was given of the maturation of cheese and the accepted views on the formation of flavour during this process. Chapters 2 and 3 describe the fractionation of the water-soluble fraction of various types of ripened cheese and the identification of volatile and non-volatile compounds that contribute to the typical cheese flavour. The formation of the volatiles from diverse precursors, e.g. amino acids and fatty acids, is also discussed. In Chapter 4 the formation of flavour from amino acids and peptides by enzymes of *Lactococcus lactis* subsp. *cremoris* B78 is described. The purification and characterization of enzymes from *Lactococcus lactis* subsp. *cremoris* B78 which are able to degrade methionine under cheese ripening conditions is reported in Chapters 5 and 6. Chapter 7 describes some preliminary experiments in which cell-free extracts of *Lactococcus lactis* subsp. *cremoris* B78 were used in combination with methionine in cheese-like model systems. A summary together with concluding remarks are given in Chapter 8.

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Chapter 2

Isolation and comparative characterization of components that contribute to the flavour of different types of cheese

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Isolation and comparative characterization of components that contribute to the flavour of different types of cheese

Summary

Water-soluble fractions (WSFs) of various types of cheese were comparatively investigated to characterize components that may contribute to cheese flavour. WSF of seven types (Cheddar, Edam, Gouda, Gruyère, Maasdam, Parmesan and Proosdij cheese) was prepared by homogenizing grated cheese with water in a stomacher and by removing remaining solids with a centrifuge. WSF was fractionated by serial ultrafiltration (with membranes of different molecular weight cut-off), followed by gel filtration and Sep-Pak C_{18} chromatography. The fractions were analysed by reversed-phase HPLC, gas chromatography, amino acid analysis and sensorically. Low-molecular-weight (<500 Da) compounds were responsible for flavour in WSF. They might be small peptides, amino acids, free fatty acids or breakdown products such compounds.

1 Introduction

The maturation of cheese and the resulting development of flavour is a very complex process. Despite extensive investigation, the compounds responsible for flavour are still largely unknown.

During cheese ripening, the main biochemical events yielding compounds that contribute to flavour are proteolysis, lipolysis and carbohydrate breakdown.

Proteolysis is governed by a variety of enzymes from different sources [35]. Chymosin is used for curd formation and in several types of cheese it contributes significantly to ripening as well. The indigenous milk proteinase plasmin is also thought to play some role in ripening [11]. The major source of proteolytic enzymes involved in cheese ripening, however, is considered to be the starter culture lactic acid bacteria [30, 33, 35]. Milk proteins (caseins) are hydrolysed by the action of these proteolytic enzymes, yielding peptides of different chain length and amino acids. These peptides and amino acids are thought to be associated directly with development of desirable [26] or undesirable [36] taste and aroma, or to act as precursors in subsequent reactions [9, 18].

The water-soluble fraction (WSF) of ripened cheese contains components that make a major contribution to the intensity of cheese flavour and it has therefore been studied extensively. Cheddar cheese has been most studied and various methods have been described to obtain WSF from this type of cheese [22, 24]. Some workers used gel filtration and HPLC to fractionate the WSF of Cheddar cheese [8, 21, 27]. Aston and Creamer [2] extensively analysed components of WSF and assessed flavour. Others have

also studied water-soluble fractions from Provolone cheese [32], Vacherin Mont d'Or cheese [25], Appenzeller cheese [3] and a Gouda-type cheese [20].

In the present study, water-soluble fractions of seven types of cheese were compared to find the contribution of their constituents to cheese flavour and flavour differentiation.

2 Materials and Methods

2.1 Cheese samples

Data about the cheeses used are summarized in Table 1. A cheese of each type was selected having a normal age for consumption.

Cheeses were tasted by five experienced cheese graders before further handling and found to be of good quality. A sector of each of the cheeses was grated (Hobart model 4812, Ohio, USA) and the grated cheese was stored at −18 °C until further investigation.

2.2 Extraction

The grated cheeses were extracted as outlined in Figure 1. WSF, designated Supernatant I, was acidified to pH 4.6 to precipitate protein and other high-molecular-weight material. The fraction soluble at pH 4.6, obtained after re-centrifugation, was designated Supernatant II. Acetic acid was removed by freeze-drying and the residue was redissolved in the original volume of distilled water. The cheese flavour intensity remained essentially unaffected by this mild treatment. The upper fat layer, Protein Pellet I, Protein Pellet II and part of the Supernatant I were stored at −18 °C until further analysis.

2.3 Fractionation

2.3.1 Ultrafiltration (UF). Freeze-dried and redissolved Supernatant II was further fractionated by ultrafiltration (Figure 1) at 4 °C in a stirred-cell type ultrafiltration module (Amicon Corporation, MA, USA), operated under a nitrogen pressure of 300 kPa using Amicon Diaflo membranes YM5 (5000 Da molecular-weight cut-off, MWCO) and YC05 (500 Da MWCO) in succession. The retentates were repeatedly washed with distilled water and refiltered to free them from the lower-molecular-weight components.

2.3.2 Gel filtration. The UF<500 fraction (see Figure 1) from Gouda cheese was separated by gel filtration on a glass column (15 mm × 820 mm) with Sephadex G-10 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) at 4 °C, using 0.01 M NaCl as an eluent (flow rate 20 ml/h, fraction volume 5 ml). Portions of 140 mg of freeze-dried UF<500 material in 1.5 ml of water were applied to the column. Absorbance was measured with an LKB 2138 Uvicord S at 280 nm (Pharmacia LKB, Uppsala, Sweden)

Table 1. Relevant data of the various cheese types.

Type	Origin	Age	Starter
Cheddar	Ireland	6-8 months	0-starter
Edam	Netherlands	4 months	L-starter
Gouda	Netherlands	6 months	DL-starter (code Bos)
Gruyère	Switzerland	4-5 months	thermophilic lactobacilli thermophilic lactococci
Maasdam	Netherlands	6 weeks	DL-starter (code Bos) propionic acid bacteria
Parmesan	Italy	2 years	thermophilic lactobacilli thermophilic lactococci
Proosdij	Netherlands	6 months	DL-starter (code Bos) non-acidifying thermophilic lactobacilli and other thermophilic organisms
Gouda 20*	Netherlands	6-12 weeks	DL-starter (code Bos)

and the elution position of salts was determined by measuring electrical conductivity (WTW LF Digi 550, Weilheim, Germany). Appropriate fractions were pooled and freeze-dried.

2.3.3 Sep-Pak C_{18} chromatography. Pre-packed Sep-Pak Plus C_{18} Environmental cartridges (Waters-Millipore, MA, USA) were used to fractionate the UF<500 material obtained from the various cheeses. Two cartridges were linked and loaded with 40 mg of freeze-dried product in 1 ml of water. The cartridges were eluted with water-ethanol by increasing the volume fraction of ethanol in four steps from 0 to 0.2. Final elution was carried out with pure ethanol. This procedure resulted in five fractions, C_{18}-1 to C_{18}-5. The eluent was removed by evaporation under reduced pressure (Büchi Rotavapor-R, Flawil, Switzerland).

2.4 Analysis

2.4.1 High-Performance Liquid Chromatography (HPLC). HPLC was carried out on a system consisting of an ISS-100 Perkin Elmer automatic sample injector, two Waters M6000 A pumps, an AGC Waters type 680 gradient controller, a Kratos 783 UV detector operating at 220 nm and a Waters model 450 UV detector operating at 280 nm. The equipment was linked to a Waters Maxima 820 data acquisition and processing system. Samples were chromatographed at 30 °C on a Bio-Rad HiPore RP-318 reversed-phase column (4.6 mm × 250 mm) preceded by a Bio-Rad C_{18} cartridge guard column.

HPLC Solvent A consisted of acetonitrile/water/trifluoroacetic acid, 50/950/1, by volume. The components were separated by a 63 min stepwise linear gradient of Solvent

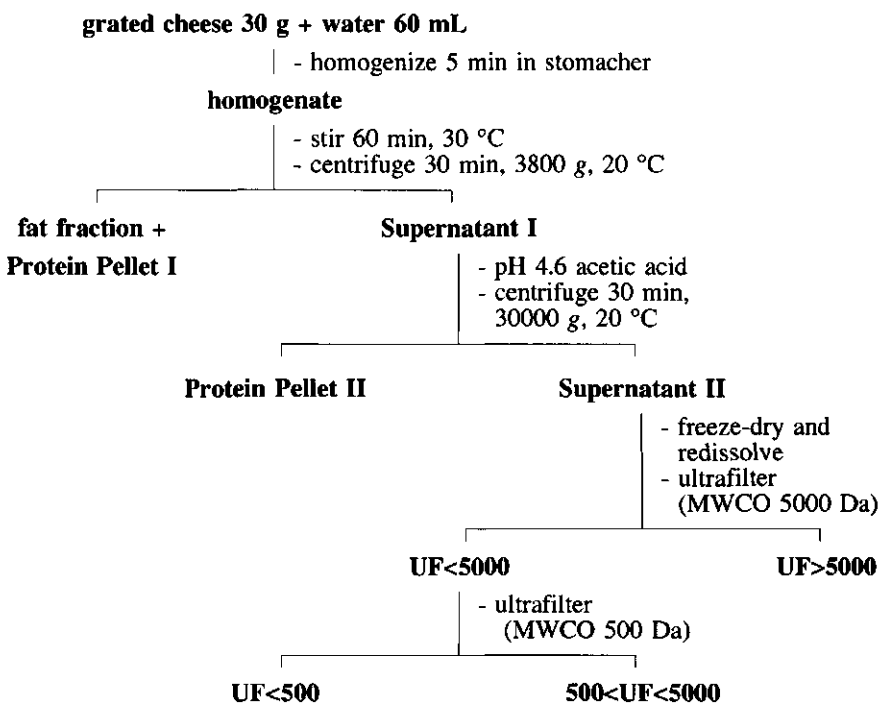

Figure 1. Extraction of grated cheese and fractionation of the extract.

B (acetonitrile/water/trifluoroacetic, 950/50/0.07, by volume) in Solvent A from 0 to 2 % Solvent B over 10 min, isocratic at 2 % Solvent B for 5 min, from 2 to 5 % Solvent B over 5 min, from 5 to 40 % Solvent B over 33 min, from 40 to 60 % Solvent B over 5 min and isocratic at 60 % Solvent B for 5 min (volume percentages throughout). The flow-rate was 0.8 ml/min.

2.4.2 Amino acid analysis. Amino acids compositions were determined on a 4151 Alpha Plus amino acid analyser (Pharmacia LKB, Uppsala, Sweden). Total amino acids were determined after hydrolysis of the samples with 6 M HCl in evacuated tubes at 110 °C for 24 hours.

2.4.3 Estimation of free fatty acids. UF<500 fractions (10 mL) were treated as described by de Jong and Badings [10] to estimate free fatty acids.

2.4.4 Sensory analysis. The various fractions were analysed by a panel of five or six experienced tasters. During round-table discussion sessions the panel members tasted 400 µl portions, placed directly onto the tongue, after two-fold dilution with distilled water of each liquid sample shown in Figure 1. The overall flavour quality and the cheese flavour intensity were scored. Overall flavour quality means the general

appreciation of the sample. During each session no more than six samples were judged. General appreciation was scored on a scale from 3 (very poor) to 8 (very good) and cheese flavour intensity on a scale from 0 (none) to 4 (very strong).

The trials were performed 'blind' and the taste of the cheese fractions was assessed taking the de-acidified Supernatant II of the corresponding cheese as reference (see Figure 1). The averages of the individual scores are presented.

Since the salt was completely transferred to the permeates during ultrafiltration, NaCl was added to the retentates in order to restore its concentration to that originally present in Supernatant II (NaCl determined by Mohr titration). Fat and protein pellets were freeze-dried and then resuspended in distilled water.

Gel filtration fractions (see 2.3.2) were tasted after removal of water by freeze-drying and subsequent dissolution of the dry material in 2 ml of distilled water. The same procedure was followed with Sep-Pak C_{18} fractions (see 2.3.3) after evaporation of the eluent.

3 Results

3.1 Fractionation and taste evaluation

Extraction of water-soluble components from cheese, and the subsequent UF steps, resulted in various fractions suitable for flavour assessment. In order not to influence the flavour of these fractions, we avoided the use of buffers and organic solvents during this fractionation. However, addition of acetic acid to precipitate proteins and subsequent freeze-drying to remove the acid were necessary (see section 2.2). Therefore, experiments were conducted to determine the effect of these treatments. Taste trials revealed that once-only acidification with acetic acid and subsequent freeze-drying (section 2.2) or rotary evaporation (section 2.3.3) of the fractions had only a very minor effect.

The fat fractions and Protein Pellets I and II (see Figure 1), after resuspending them in distilled water, were organoleptically assessed. The Protein Pellets were always almost tasteless and odourless, whereas the fat fractions possessed a buttery aroma. The flavour of the water-soluble Supernatant II fractions of all the cheeses was described as cheese-like. Moreover, the taste panellists recognized some of the Supernatant II fractions as originating from the corresponding cheese-types i.e. Gruyère, Gouda and Parmesan.

Table 2. Average flavour scores of water-soluble fractions. For definition of the scales see section 2.4.4.

Cheese type	Fraction									
	Supernatant II		UF>5000		UF<5000		500<UF<5000		UF<500	
	overall flavour	cheese flavour	overall flavour	cheese flavour	overall flavour	cheese flavour	overall flavour	cheese flavour	overall flavour	cheese flavour
Cheddar	6.8	1.2	5.2	0.2	6.3	1.0	5.5	0.1	6.5	1.0
Edam	6.1	0.5	5.3	0	6.1	0.6	5.6	0.2	5.7	0.3
Gouda	6.5	1.0	5.1	0	6.6	0.9	5.3	0.2	6.2	0.8
Gruyère	7.0	1.5	4.8	0.2	6.5	1.5	5.3	0.3	6.5	1.3
Maasdam	6.7	1.4	5.3	0.3	6.5	1.4	5.5	0.4	6.2	1.1
Parmesan	7.0	1.5	5.6	0.2	6.9	1.4	5.3	0	6.6	1.3
Proosdij	6.5	1.5	5.1	0.4	6.5	1.6	5.2	0.3	6.4	1.4

The highest average values among water-soluble fractions for overall flavour quality and cheese flavour intensity (Table 2) were scored for the UF permeates, except for the fraction originating from Edam cheese.

The UF<500 fraction from Gouda cheese was further fractionated by Sephadex G-10 gel filtration. Because the fractions collected were to be organoleptically tested, the use of salt in the eluent was minimized. As a consequence, some ionic interaction between sample components, e.g. tryptophan, and column material remained, and separation was therefore not strictly on the basis of molecular size. Figure 2 shows a gel filtration pattern of the UF<500 fraction of Gouda cheese. The results of conductivity measurements, which are indicative of the elution of salts, are also shown.

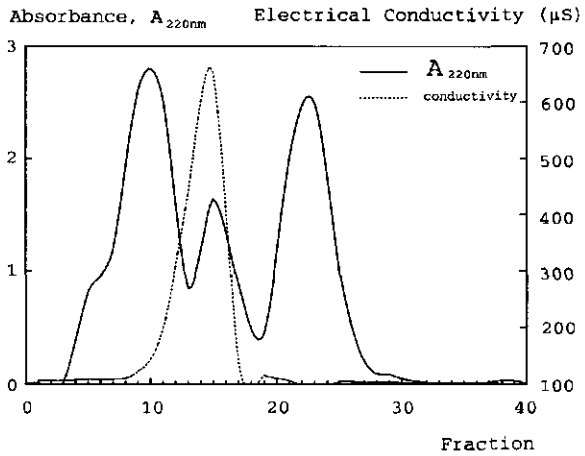

Figure 2. Sephadex G-10 gel filtration chromatogram of the UF<500 fraction from Gouda cheese.

Sensory analysis followed solution of the freeze-dried material in distilled water (Table 3) and showed the cheese-like flavour mainly in one pool, comprising column fractions 9 to 13. These fractions contained approximately 25 % of the salt present in the UF<500 fraction loaded onto the column (total salt content in the UF<500 fraction from Gouda cheese: 7 g/L)(Figure 2). The pooled fractions 1 to 8 lacked cheese flavour, even after the addition of NaCl to 7 g/L.

Table 3. Results of sensory analysis on Sephadex G-10 gel filtration pools originating from Gouda cheese. For definition of scales see section 2.4.4.

Combined fractions	Overall flavour quality	Cheese flavour intensity
UF<500 (reference)	6.5	1.0
G-10 1 to 8	4.8	0.2
G-10 9 to 13	6.8	1.5
G-10 14 to 19	4.2	0.2
G-10 20 to 24	5.8	0.2
G-10 25 to 30	4.8	0

Besides gel filtration (only on the Gouda cheese UF<500 fraction), Sep-Pak C_{18} chromatography was used to fractionate the UF<500 material from various types of cheese. Table 4 shows the results of the taste trials with fractions from Gouda cheese. As can be seen, only the first eluting fraction possessed a cheese flavour. The same results were obtained for Cheddar, Parmesan, Gruyère, Proosdij and Maasdam (results not shown). UF<500 from Edam cheese was not further fractionated.

Table 4. Results of sensory analysis on Sep-Pak C_{18} fractions originating from Gouda cheese. For definition of scales see section 2.4.4.

Fractions	Overall flavour quality	Cheese flavour intensity
UF<500 (reference)	6.5	1.0
C_{18}-1	7.0	2.0
C_{18}-2	4.5	0
C_{18}-3	5.0	0
C_{18}-4	5.0	0
C_{18}-5	5.0	0

3.2 HPLC and analysis for amino acids

HPLC patterns for UF<500 fractions were similar for all the cheeses (Figure 3). The The composition of the main peaks was determined by amino acid analysis after collecting the peak material at the outlet of the UV detector. The peaks at 6.7 min, 11.7 min and 25.2 min were due to tyrosine, phenylalanine and tryptophan, respectively.

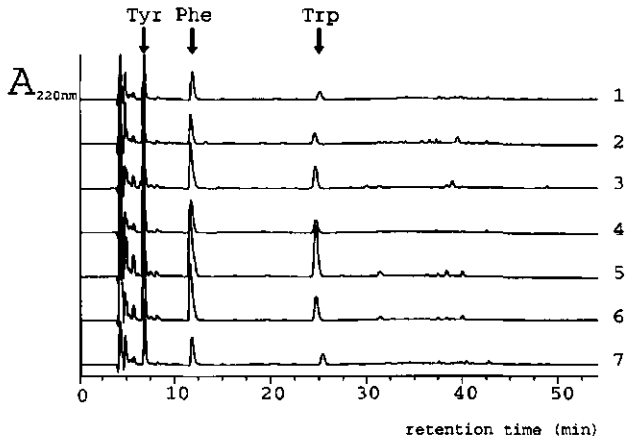

Figure 3. Reversed-phase HPLC chromatograms of UF<500 fractions from cheeses. Types of cheese: 1. Cheddar, 2. Edam, 3. Gruyère, 4. Maasdam, 5. Parmesan, 6. Proosdij, 7. Gouda.

Because most amino acids do not exhibit sufficient absorbance at 220 nm, UF<500 fractions of the various cheeses were analysed for amino acids. The amounts of free and total amino acids were measured (Figure 4). In all the UF<500 fractions, free amino acids were the main components. The individual amino acid profiles (not shown) were similar for all the cheeses. Glutamic acid, leucine and phenylalanine were the major amino acids in both the free and the total amino acid fractions. Valine, proline and lysine were also quite abundant. Notable was the presence of non-casein amino acids like γ-amino butyric acid and ornithine in the UF<500 fractions. The concentration of total amino acids was higher than of total free amino acids in all the samples, indicating the presence of peptides (Figure 4). The small peaks between 30 and 45 min in the chromatograms of Figure 3 are probably due to these peptides.

Maasdam, Cheddar, Gouda and Edam cheese contained approximately equal amounts (g/l) of free amino acids in the UF<500 fraction (Figure 4). For Gouda and Edam cheese, the concentration of total amino acids in the UF<500 fraction was somewhat higher than for Maasdam and Cheddar cheese. UF<500 fractions from Gruyère, Proosdij and especially Parmesan cheese contained the highest concentrations of total amino acids. The proportion of total free amino acids in the UF<500 fractions of these cheeses

was, however, slightly smaller (Figure 4), indicating the presence of somewhat higher concentrations of small peptides.

Reversed-phase HPLC profiles of UF-fractions from Gouda and Proosdij cheese (both approximately 6 months old) were compared (Figure 5). The 220 nm absorbance profile of the 500<UF<5000 fraction from Proosdij cheese (made with lactococci and lactobacilli) (trace 2 in Figure 5) differed markedly from that of the corresponding frac-

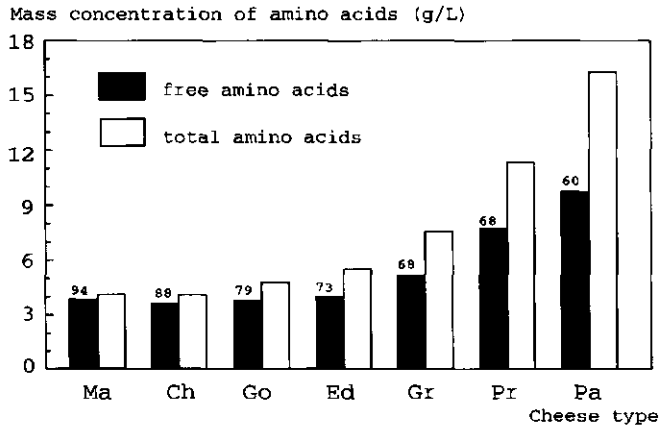

Figure 4. Total free and total (free+peptide bound) amino acids in UF<500 fractions from cheeses. Values above columns: free amino acids as percentage of total amino acids. Types of cheese: Ma: Maasdam, Ch: Cheddar, Go: Gouda, Ed: Edam, Gr: Gruyère, Pr: Proosdij, Pa: Parmesan.

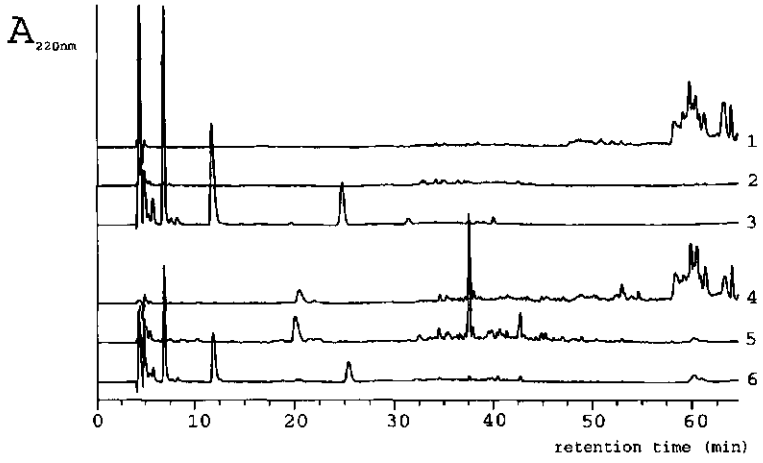

Figure 5. Reversed-phase HPLC chromatograms of fractions from Gouda and Proosdij cheese. Fractions: 1. UF>5000 Proosdij, 2. 500<UF<5000 Proosdij, 3. UF<500 Proosdij, 4. UF>5000 Gouda, 5. 500<UF<5000 Gouda, 6. UF<500 Gouda.

tion from Gouda cheese (made with lactococci only) (trace 5), particularly in the 30 to 45 min region, where larger peptides eluted. Compared with the situation in Gouda cheese, the breakdown of these larger peptides occurs at a relatively high rate in Proosdij cheese.

The combined Sephadex G-10 fractions 9 to 13 from Gouda cheese (Figure 2) contained approximately 50 % of the peptide material of the UF<500 fraction (Figure 6), as calculated from the free/total amino acid ratio. The combined fractions 1 to 8 completely lacked cheese flavour. Furthermore, the removal of this 1-8 pool (Figure 6A) from the UF<500 fraction had no discernible effect on the cheese flavour of the remaining material represented by the 9-13 pool of Figure 6B.

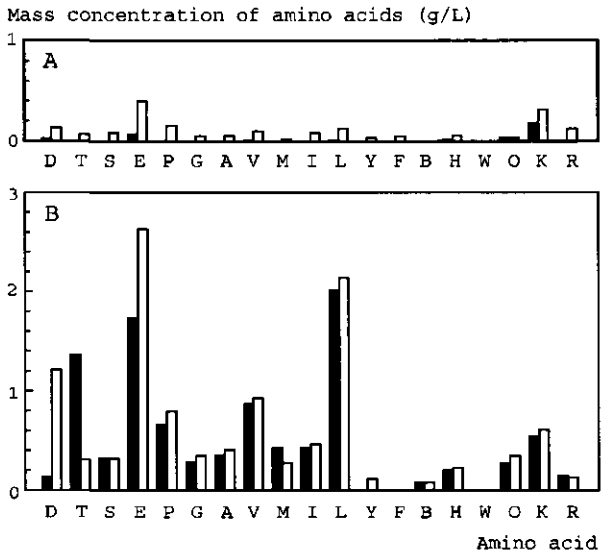

Figure 6. Mass concentration of free (filled bars) and total (free+peptide-bound)(open bars) amino acids in Sephadex G-10 Fractions 1 to 8 (A) and Fractions 9 to 13 (B) from Gouda cheese.
One-letter codes for the notation of amino acids were used [19]. B, γ-aminobutyric acid and O, ornithine. Note that the concentration of free T includes that of free Q and N (co-eluting on the column) and that the respective concentrations of total E and D include total Q and N which are converted by acid hydrolysis.

Reversed-phase analysis of Sep-Pak C_{18} fractions from UF<500 material of Gouda cheese revealed that only strongly hydrophilic peptides (retention time <10 min) were present in the fraction with cheese flavour (C_{18}-1, see Figure 7). These peptides represented about 10 % of the total amount of amino acids present in fraction C_{18}-1. More hydrophobic peptides (retention time >10 min) were possibly present in subsequent C_{18} fractions which, however, lacked flavour.

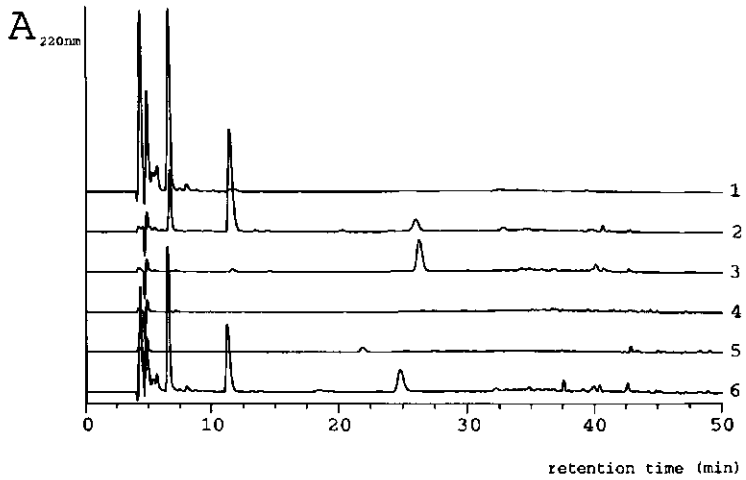

Figure 7. Reversed-phase HPLC chromatograms of Sep-Pak C_{18} fractions from Gouda cheese. Fractions: 1. C_{18}-1, 2. C_{18}-2, 3. C_{18}-3, 4. C_{18}-4, 5. C_{18}-5, 6. UF<500.

3.3 Analysis for free fatty acids

Besides amino acids and peptides, volatile compounds, such as fatty acids, occur in WSF of cheeses and therefore in the UF<500 fractions. The presence of volatile compounds in these fractions became especially evident during sensory analyses. Almost all of the samples with a cheese-like taste possessed a 'cheesy' odour as well.

Only relatively short-chain fatty acids (<C9) were present in the UF<500 fractions (Table 5). Gruyère, Maasdam and Parmesan cheese contained the highest amounts of free fatty acids, C3 and C4 being the major representatives. For comparison, free fatty acids were also determined in the UF<500 fraction of a low-fat cheese (Gouda, 20+).

4 Discussion

WSF makes a major contribution to the intensity of the cheese flavour [2, 24]. Several studies have been devoted to the fractionation and analysis of the WSF from Cheddar cheese [2, 8, 21, 27]. Our study revealed that, except in the case of Edam cheese, UF<500 fractions of cheeses contained the components responsible for cheese flavour. This finding agrees with those of other workers on the fractionation of Cheddar cheese [2] and Comté cheese [31], in which components <1000 Da and <500 Da, respectively, were considered to be responsible for cheese flavour. The peptides and proteins in the

Table 5. Free fatty acids in UF<500 fractions from various types of cheese. Concentrations in μM. Abbrevations: C3:0: propionic acid, C4:0: butyric acid, C4:0 iso: isobutyric acid, C5:0 iso: isovaleric acid, C6:0: caproic acid, C8:0: caprylic acid.

Cheese type	C3:0	C4:0	C4:0 iso	C5:0 iso	C6:0	C8:0	sum C3-C8
Cheddar	8	73	0	0	7	0	88
Edam	19	64	35	93	10	0	221
Gouda	13	146	7	22	17	0	205
Gruyère	1345	1716	276	398	47	1	3783
Maasdam	9122	67	6	45	12	1	9253
Parmesan	46	1156	1	7	134	3	1347
Proosdij	15	114	4	10	20	1	164
Gouda 20*	8	21	5	19	5	0	58

UF>500 fractions do not contribute directly to the actual flavour of the cheeses. The deviant results for Edam are not fully explicable. Although the protein pellets from this cheese were devoid of a cheese-like flavour, association of flavour components with proteins or larger peptides could have been responsible for the loss of flavour of the WSF. However, repeated extraction of these pellets with distilled water did not result in WSF with cheese flavour.

The UF<500 fractions of the various cheeses include low-molecular-weight peptides (probably not larger than tetrapeptides), amino acids and further breakdown products. Their formation results from the action of enzymes from milk and lactic acid bacteria [12, 35]. The larger amounts of peptides and free amino acids in the UF<500 fractions of Gruyère, Proosdij and Parmesan cheese compared with those in the corresponding fractions of the other cheeses are probably due to the action of lactobacilli, which can produce larger amounts of amino acids than lactococci [5, 23]. For Parmesan cheese, the relatively high age also contributes to the higher concentration of free amino acids. The lactobacilli are responsible for a higher rate of breakdown of larger peptides in 500<UF<5000 fractions of Gruyère, Proosdij and Parmesan cheese. The higher production rate of free amino acids in these cheeses may, together with other properties of the lactobacilli, account for the considerable difference in flavour characteristics. Although free amino acids are present in the fractions with a cheese-like flavour (UF<500), their direct contribution to the actual cheese flavour is probably limited [28]. Free amino acids more likely act as precursors for cheese flavour compounds; both enzymic and non-enzymic processes may contribute to their break-down [6, 35]. So there is not necessarily a relation between cheese flavour and total free amino acid concentration, which is further illustrated by differences in flavour between cheeses with almost the same concentrations of free amino acids (Cheddar, Maasdam, Gouda, see Figure 4). The presence of non-casein amino acids, such as γ-aminobutyric acid and

ornithine, in the UF<500 fractions can be ascribed to enzymic conversion of glutamic acid and arginine, respectively [14, 32, 37].

The role of peptides in the UF<500 fractions in relation to flavour is not clear. The pooled Fractions 1 to 8 of the Sephadex G-10 separation, and the Sep-Pak fractions C_{18}-2 to C_{18}-5 containing mainly small peptides, lacked cheese flavour (see typical examples in Tables 3 and 4). Several authors mention small peptides as direct flavour components in cheese [15, 25, 26]. Our results with the cheesy flavoured fractions 9 to 13 of the Sephadex G-10 separation and the Sep-Pak C_{18}-1 fraction of various cheeses indicate that the direct contribution of small peptides to the actual cheese flavour probably is limited.

Small peptides and amino acids must be mainly responsible for basic flavours (e.g. brothy [2], savoury [7], sweet [4, 17], bitter [36], salty), on which actual cheese flavours are superimposed after their formation from amino acids by enzymic and/or chemical pathways.

Only shorter-chain fatty acids (<C9) were present in the UF<500 fractions. They could influence flavour more than larger fatty acids (>C8) [29]. The larger fatty acids, which are certainly present in cheese, are most likely retained in the fat layer during extraction.

Parmesan and Gruyère cheese contained relatively high amounts of free fatty acids. In these cheeses, butyric acid (C4:0) probably plays an important role in flavour [6]. The high concentration of propionic acid (C3:0) in Maasdam cheese develops by the action of propionic acid bacteria. UF<500 fractions from Gouda, Edam, Proosdij, Cheddar and Gouda 20+ cheese contained much less free fatty acids than those of Parmesan and Gruyère, and so the contribution of fatty acids to flavour will be much less.

Free fatty acids are formed in cheese by lipolysis of fats [13, 16, 34] and through catabolism of lactic acid and amino acids by bacteria (mainly short-chain free fatty acids like C3 and C4) [1, 6, 34]. The larger amounts of C4:0 in Parmesan and Gruyère cheese could also originate from butyric acid fermentation (late blowing).

5 Acknowledgements

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Chapter 3

A comparative study of volatile compounds in the water-soluble fraction of various types of ripened cheese

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A comparative study of volatile compounds in the water-soluble fraction of various types of ripened cheese

Summary

Volatile constituents present in the water-soluble fraction (WSF) of eight hard-type cheeses (Cheddar, Edam, Gouda, Gouda 20+, Proosdij, Maasdam, Gruyère and Parmesan) were isolated and identified by using a dynamic headspace technique. The compounds were isolated from the WSF of cheeses by purge-and-trap extraction and analysed by gas chromatography-mass spectrometry. Fifty-three components were identified and 25 were quantitatively determined. The volatiles belonged to six major groups: fatty acids, esters, aldehydes, alcohols, ketones and sulphur compounds. Most of them were present in the WSF of all eight types of cheese studied. The aroma characteristics of the volatiles and their possible formation from compounds like fats, lactose and amino acids, are described.

1 Introduction

The flavour of cheese originates from microbial, enzymic and chemical transformations. The breakdown of milk proteins, fat, lactose and citrate during ripening gives rise to a series of volatile and non-volatile compounds which may contribute to cheese flavour. Proteolysis, by enzymes from milk, rennet and microorganisms, is a major biochemical event. Other factors, such as lipolysis and lactose fermentation, also play an important role [1].

Previous research has shown that the water-soluble fraction (WSF) of ripened cheese contains components that make a major contribution to flavour [4, 18, 26, 45]. Non-volatile compounds, like peptides and amino acids, present in the WSF of cheeses have been studied extensively [13, 34, 38]. From work performed at our laboratory it became clear that the direct contribution of peptides and amino acids to cheese flavour is probably limited [18]. A similar conclusion regarding the limited role of amino acids can be drawn from studies by Aston and Creamer [4]. More than amino acids and peptides, other compounds seem essential for true cheese flavour. The formation of these, often volatile, compounds occurs concurrently with proteolysis, hydrolysis of fats and carbohydrate breakdown.

Both enzymic and non-enzymic modification pathways have been suggested for the formation of volatile flavour compounds from amino acids, free fatty acids and lactic acid in cheese. Enzymes from microorganisms present in cheese that are involved in the degradation of amino acids include deaminases, decarboxylases, transaminases and enzymes converting amino acid side chains [27]. Amongst the products formed are amines, aldehydes, alcohols, acids and sulphur compounds [10, 11, 12, 23, 45]. Volatile

products formed via non-enzymic reactions involving amino acids have also been reported [21, 30]. In the case of breakdown of fatty acids, the predominant breakdown products are esters, methyl ketones and secondary alcohols [12, 22, 41]. The flavour attributes of the compounds formed range from pleasant-fruity for esters to putrid-unclean for sulphur compounds.

The volatile aroma components of various cheeses have received a great deal of attention and a large number of volatiles have been detected in individual types of cheese. Various methods have been applied to the extraction of the volatiles from WSF and subsequent gas-chromatographic analysis of the compounds. Banks *et al.* [6] used steam distillation and gas chromatography-mass spectrometry (GC-MS) for extraction and identification of volatile compounds from Cheddar cheese. The use of high-vacuum distillation, combined with gas-chromatographic analysis, has also been reported [19, 33]. Headspace techniques [8, 47, 48] are used frequently nowadays because (steam) distillation methods, or other extraction procedures, often tend to suffer from artefact formation and decomposition of components [31, 47].

The objective of the present study was to compare the relative amounts of a number of volatile compounds present in the WSF of a series of cheeses. This paper describes the isolation and identification of these compounds in the WSF of eight hard-type cheeses. The procedure used for preparation of WSF was essentially the same as the method applied previously in order to relate the results of the present work with results of sensorical tests [18]. A dynamic headspace technique, followed by GC and GC-MS, was used for this purpose [36, 37]. The possible origin of the identified volatiles and the consequences of their occurrence in the various cheeses are discussed.

2 Materials and Methods

2.1 Cheese samples and preparation of WSF

The following cheeses were used: Cheddar (age 6-8 months, 330 g fat kg^{-1} cheese) from Ireland; Edam (4 months, 243 g fat kg^{-1} cheese), Gouda (6 months, 308 g fat kg^{-1} cheese), Gouda 20^{+} (6-12 weeks, 120 g fat kg^{-1} cheese), Proosdij (6 months, 308 g fat kg^{-1} cheese) and Maasdam (6 weeks, 285 g fat kg^{-1} cheese) from The Netherlands; Gruyère (4-5 months, 350 g fat kg^{-1} cheese) from Switzerland; Parmesan (2 years, 270 g fat kg^{-1} cheese) from Italy. The fat contents given are the amounts that are found normally for these types of cheese. All cheeses were of normal age for consumption and of good organoleptic quality.

For the preparation of WSF, a sector of each of the cheeses was grated (Hobart model 4812, Ohio, USA). Grated cheese was mixed with bidistilled water (1:2, w:v) and homogenized in a stomacher (Seward Stomacher 400 Lab Blender, London, UK) for 5 min. WSF was obtained after removal of protein and fat material by centrifugation for 30 min at 15000 *g* and 4 °C. Before performing gas chromatography, internal standards (methyl butyrate and methyl pentanoate, 99 % purity, Fluka Chemika, Buchs,

Switserland) and the anti-foaming agents tetradecanol (97 % purity, Fluka Chemika) and a mixture of cyclic siloxanes (Silbione, Rhone-Poulenc, France, purified by repeated vacuum treatment) were added to the WSF.

2.2 Purge-and-trap procedure

For the identification and quantification of volatile components in the WSF, purge-and-trap thermal desorption cold-trap (TDCT) gas chromatography was used. A 20 mL aliquot (for both gas chromatography-mass spectrometry, GC-MS, and quantitative gas chromatography with flame ionisation detection, GC-FID) of WSF was purged with 150 mL min^{-1} helium for 30 min at room temperature. Volatile components were collected on a trap containing Carbosieve SIII (10 mg, 60-80 mesh, Supelco, Belle Fonte, PA, USA) and Carbotrap (80 mg, 20-40 mesh, Supelco, Belle Fonte, PA, USA). The trapped components were transferred onto the column of a gas chromatograph using a Chrompack PTI injector (Chrompack, Middelburg, the Netherlands) in the TDCT mode, by heating the trap for 10 min (250 °C) [5, 37]. A narrow injection band of the components was achieved by cryofocussing (-100 °C).

2.3 Gas Chromatography-Mass Spectrometry (GC-MS)

The gas chromatograph (Hewlett Packard HP 5890A) was equipped with a 60 m × 0.32 mm i.d. fused-silica capillary DB1 column (film thickness 1.0 µm, J&W Scientific, Folsom, CA, USA) and the injector-block temperature was 200 °C. The carrier gas was helium (column pressure 80 kPa). The oven-temperature programme was: 1 min at 40 °C, 10 °C min^{-1} to 50 °C, 1 min at 50 °C, 1 °C min^{-1} to 60 °C, 10 °C min^{-1} to 250 °C and 15 min at 250 °C. The GC column was connected directly to the ion source (temperature 200 °C) of a VG 12-250 (Micromass, Altrincham, UK) quadrupole mass spectrometer (temperature of interface line: 210 °C). The mass spectrometer was operating in the scanning mode from 25 to 500 Da at 1 scan s^{-1}. Ionisation was by electron impact at 70 eV. Structures were assigned by spectrum interpretation and comparison of the spectra with bibliographic data (NIST/EPA/MSDC Mass Spectral Database, T.G. House, Cambridge, UK).

2.4 Quantification of volatile components by gas chromatography (GC-FID)

Gas-chromatographic quantification of components in the WSF of cheeses was performed using a Carlo Erba MEGA 5360 GC gas chromatograph equipped with a flame ionisation detector (FID 40, Carlo Erba, Milan, Italy). The purge-and-trap procedure, as well as the GC-FID analyses, were performed in triplicate. Column and chromatographic conditions (column pressure and temperature programme) were the same as those described for GC-MS.

Identification of compounds was achieved on the basis of retention times as determined by GC-MS and by comparison of the GC retention times with the retention times of authentic compounds. For quantification the average peak areas and standard deviations were determined.

3 Results and Discussion

The (bio)chemical transformations which occur during the ripening of cheese are highly complex. Although lactose fermentation and protein and fat hydrolysis are recognised as primary ripening processes, further breakdown of the products formed will give rise to new compounds. Several types of degradations occur simultaneously and the ultimate result will be a very wide range of, often volatile, compounds. The precise contribution of these volatile compounds to the flavour of cheeses is largely unknown.

During the present study, we identified 53 volatile components in the WSF of the eight cheeses investigated. In Tables 1 - 5, the volatile compounds tentatively identified by GC-MS are presented, as well as the relative amounts of those compounds quantified by GC-FID. The volatiles belonged to six major groups: fatty acids, esters, aldehydes, alcohols, ketones and sulphur compounds. Most of the compounds were present in all cheeses. Their concentrations, however, showed distinct differences. Comparison of relative amounts was possible only for homologous compounds because of differences in the yield of the purge-and-trap process, which undoubtedly occurred as a result of variance in, e.g., hydrophilicity, polarity and volatility of the various components in the WSF, and differences in the FID response.

3.1 Fatty acids and esters

Several fatty acids were detected in the WSF from the cheeses (Table 1). Determination of peak areas was, however, difficult because of asymmetrically shaped peaks, due to the type of column used in GC. Therefore, only qualitative data concerning fatty acids and some esters are presented in this paper. However, previous research at our laboratory, using a more suitable technique for quantification of fatty acids, has shown that butyric acid was present at relatively high concentrations in Gruyère and Parmesan cheese (Table 5 in [18]). High concentrations of propionic acid were present in Gruyère and, particularly, Maasdam cheese (Table 5 in [18]).

During the ripening of cheese, free fatty acids containing four carbons or more can originate from the lipolysis of milk fat or from breakdown of amino acids [44]. Lipolysis may be due to the action of the indigenous lipase of milk in cheeses made from raw milk (Parmesan, Gruyère), or to the action of microbial lipases [12]. Lactic acid bacteria present in starter cultures are generally only weakly lipolytic. Moulds, present in many French cheese-types, e.g. Roquefort, however can produce large quantities of lipolytic enzymes [12, 35]. The role of lipolytic activities from microorganisms, such as *Brevibacterium linens*, in surface-ripened cheeses, e.g. Gruyère,

is uncertain. From lactose fermentation, via lactate, some short-chain fatty acids such as acetic acid and propionic acid may be formed [7, 20, 42].

Fatty acids are important components in the flavour of many cheese types. Short-chain fatty acids impart a desirable peppery taste to blue cheese flavour [43]. Numerous short and medium-chain fatty acids, each with a characteristic flavour note, have been found in Camembert cheese [35]. In Parmesan and Swiss Gruyère cheese [9], acetic and butyric acid probably play a significant role in flavour. Large amounts of butyric acid, which might originate from butyric acid fermentation, are nevertheless undesirable. The high concentration of propionic acid in Maasdam cheese, produced by the action of propionic acid bacteria, results in a slightly sweet flavour, as is the case for Swiss Emmental cheese [42].

The role of fatty acids as precursors for other flavour compounds is also of importance. Esters (Table 1), but also methyl ketones and secondary alcohols (discussed below), are formed from fatty acids. Esterification takes place by an enzymic or chemical reaction of fatty acids with primary alcohols [9, 12, 35]. Many esters have a sweet-fruity aroma. Especially, ethyl esters are known for their important role in the formation of a fruity character in cheeses [3, 7]. In accordance with this, high concentrations of ethyl butanoate were found in the fruity tasting cheeses Gruyère, Parmesan and Proosdij.

3.2 Aldehydes

Aldehydes were quantitatively a major class of volatile components in the WSF of the cheeses investigated (Table 2). Branched aldehydes probably originate from amino acid degradation. In this respect enzymic processes, by enzymes from cheese microorganisms [32, 33], as well as non-enzymic processes, e.g. the Strecker degradation [21], were reported previously. In Cheddar cheese 2-methyl propanal, 2-methyl butanal and 3-methyl butanal, produced from valine, isoleucine and leucine, respectively, were responsible for unclean and harsh flavours [17]. We found high concentrations of 3-methyl butanal in Parmesan and Proosdij cheese and in the latter the aldehyde is responsible for a typical spicy, chocolate-like flavour [R. Neeter, unpubl. results]. Griffith and Hammond [21] reported malty aromas in connection with aldehydes produced from the branched aliphatic amino acids valine, isoleucine and leucine.

Straight-chain aldehydes, such as butanal, pentanal, hexanal, heptanal and nonanal, were detected in the WSF of all cheeses. These aldehydes are formed during ß-oxidation of unsaturated fatty acids [14, 28, 33]. WSF of Gruyère and Parmesan cheese, with a high occurrence of lipolysis, contained relatively high concentrations of linear aldehydes. They are characterised by green-grass-like and herbaceous aromas [33].

Acetaldehyde is produced in yoghurt during lactose metabolism by lactic acid bacteria, but also by breakdown of threonine [25, 35]. The latter process could also be of importance during cheese ripening.

Table 1. Acids and esters in the water-soluble fraction of eight cheese types[a].

Acids and Esters	Cheese type															
	Gouda 20+		Gouda		Proosdij		Gruyère		Maasdam		Edam		Parmesan		Cheddar	
	Area[b]	SD(%)[c]	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)
Acetic acid	*		*		*		*		*		*		*			
Propanoic acid							*		*							
Butyric acid					*		*		*		*		*		*	
Pentanoic acid							*									
Hexanoic acid	*		*		*		*		*		*		*		*	
3-Methyl butanoate							*									
Ethyl acetate	*		*		*		*		*		*		*		*	
Ethyl butanoate	65	7	87	4	161	10	219	18	*		96	6	166	11	93	9
Ethyl hexanoate	*		*		*		*		*		*		*		*	
Methyl acetate	*		*		*		*		*		*		*		*	

[a] Tentative identification by spectrum interpretation and by comparison of the spectra with bibliographic data.
[b] Peak area (mean value of triplicate GC-FID analysis); arbitrary units were used (*: compound identified by GC-MS only).
[c] Standard deviation (SD) of triple determinations.

Table 2. Aldehydes in the water-soluble fraction of eight cheese types[a].

Aldehydes	Cheese type															
	Gouda 20+		Gouda		Proosdij		Gruyère		Maasdam		Edam		Parmesan		Cheddar	
	Area[b]	SD(%)[c]	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)
2-Butenal					*											
2-Methyl-butanal					*				*							
2-Methyl-propenal	*		*		*		*		*		*		*		*	
2-Methyl-propanal	*		*		*		*		*		*		*		*	
3-Methyl-butanal	74	18	352	18	1107	26	425	5	440	20	212	18	663	4	174	31
Acetaldehyde	70	48	142	56	117	22	119	20	47	20	74	43	135	11	181	25
Benzaldehyde	*		*		*		*		*		*		*		*	
Butanal	*		51	19	43	17	31	27	*		9	19	33	25	*	
Pentanal	120	12	188	10	228	19	337	18	233	19	39	9	122	9	153	39
Hexanal	213	20	174	6	214	26	326	5	*		102	9	540	5	205	26
Heptanal	63	9	143	17	132	20	275	8	107	15	44	1	299	14	146	6
Nonanal	132	9	163	15	179	23	148	24	117	5	94	12	235	19	127	14

[a] Tentative identification by spectrum interpretation and by comparison of the spectra with bibliographic data.
[b] Peak area (mean value of triplicate GC-FID analysis); arbitrary units were used (*: compound identified by GC-MS only).
[c] Standard deviation (SD) of triple determinations.

3.3 Alcohols

Primary alcohols are reported to be present in various cheese types, e.g. Parmesan [7], Cheddar [1], Roquefort [19] and Domiati [14]. They are considered to originate from the corresponding aldehydes following a reaction pathway involving alcohol dehydrogenases. The strong reducing conditions in hard cheeses may favour the formation of alcohols from aldehydes. During our study, large amounts of ethanol were detected in the WSF of the cheeses (Table 3). Ethanol was probably formed from acetaldehyde by lactic acid bacteria [25]. Ethanol, but also other aliphatic primary alcohols present in WSF (e.g. 1-butanol, 1-pentanol and 1-hexanol), may impart a fruity, nutty note to the flavour of cheese [19]. In certain cheeses, e.g. Gouda and Cheddar, high levels of these alcohols could be responsible for flavour defects. The presence of the branched-chain primary alcohols 2-methyl-1-butanol, 2-methyl-1-propanol and 3-methyl-1-butanol, indicates conversion of the aldehydes produced from isoleucine, valine and leucine respectively. 3-Methyl-1-butanol, present at substantial levels in WSF of Maasdam, Edam and Parmesan cheese (Table 3), has a pleasant aroma of fresh cheese [33].

Secondary alcohols (Table 3) are formed in cheeses by enzymic reduction of methyl ketones, which themselves are produced from fatty acids [12, 35]. These alcohols are typical components of the flavour of blue cheeses [16]. In Cheddar cheese, however, production of 2-propanol from acetone has been reported, as well as the production of 2-butanol from butanone [44]. Butanone originated from diacetyl, probably by action of non-starter bacteria.

Phenol, a major flavour compound in surface-ripened cheeses, was detected in the WSF of all cheeses (Table 3). It is formed during the microbiological breakdown of tyrosine [39].

3.4 Ketones

Ketones are common constituents in most dairy products. Nine different ketones, mainly methyl ketones, were identified in the WSF of the cheeses investigated (Table 4). Methyl ketones are primarily recognised for their contribution to the flavour of mould-ripened cheeses, such as blue cheese [46]. The characteristic aroma of blue cheeses, e.g., Roquefort, as well as Camembert, is frequently attributed to 2-heptanone and 2-nonanone [19, 35, 43, 44]. The significance of methyl ketones for the flavour of other cheeses is not yet established completely. 2-Pentanone may, however, impart an orange-peel aroma to Cheddar cheese [3]. In Parmesan [7] and Mozzarella [33] cheese, methyl ketones are also thought to play an important role as flavour constituents. In our study, unsaturated methyl ketones were not detected; their presence in cheeses has, however, been reported [7, 19].

Methyl ketones are formed in cheese by enzymic oxidative decarboxylation of fatty acids (β-oxidation pathway) [1, 14, 46]. Due to the (reducing) cheese environment,

Table 3. Alcohols in the water-soluble fraction of eight cheese types[a].

Alcohols	Cheese type													
	Gouda 20+		Gouda		Proosdij		Gruyère		Maasdam		Edam		Parmesan		Cheddar	
	Area[b]	SD(%)[c]	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)
Butan-1,3-diol	*		*													
1-Butanol	262	18	234	13	276	5	719	2	483	14	234	8	789	16	151	19
1-Pentanol	65	21	117	12	83	7	183	5	97	24	49	18	91	19	215	14
2-Butanol	83	22	25	31	67	13	283	2	109	4	1070	5	593	19	456	12
2-Heptanol															*	
2-Methyl-1-butanol	36	13	48	14	72	19	213	4	580	10	48	11	210	17	36	35
2-Methyl-1-propanol	99	17	171	5	159	12	151	3	152	8	156	4	*		100	3
2-Pentanol	*		*		*		*				*		*			
2-Propanol	50	43	146	29	88	10	444	21	145	19	168	9	1652	12	194	94
3-Methyl-1-butanol	261	14	262	9	346	13	301	4	590	16	525	5	442	22	67	29
3-Methyl-3-buten-1-ol	25	10	23	13	18	24	22	1	*		15	14	67	12	180	16
Methanol	30	38	58	9	39	12	763	6	184	16	62	7	537	24	111	64
Ethanol	>6000		>6000		>6000		>6000		>6000		>6000		>6000		>6000	
Hexanol	*		*		*		*		*		*		*		*	
Phenol	*		*		*		*		*		*		*		*	

[a, b, c] See Table 4.

Table 4. Ketones in the water-soluble fraction of eight cheese types[a].

Ketones	Cheese type													
	Gouda 20+		Gouda		Proosdij		Gruyère		Maasdam		Edam		Parmesan		Cheddar	
	Area[b]	SD(%)[c]	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)
Acetophenone	*		*		*		*		*		*		*		*	
Pentan-2,3-dione	17	12	19	36	27	15	22	8	58	15	12	12	*		*	
2-Pentanone	112	14	75	5	44	3	243	3	51	20	211	4	63	5	67	22
2-Heptanone	129	3	226	11	241	16	219	2	119	7	513	6	223	11	188	15
2-Nonanone	35	13	35	20	243	8	115	13	158	20	74	8	280	14	102	12
Acetoin	*		*		*				*							
Acetone	1354	56	335	29	394	13	440	15	422	13	517	7	627	17	282	75
Butanone	1020	21	345	4	284	14	232	7	249	8	1349	4	291	7	222	13
Diacetyl	>6000		3538	43	2411	11	72	15	>6000		332	11	28	11	187	123

[a] Tentative identification by spectrum interpretation and by comparison of the spectra with bibliographic data.
[b] Peak area (mean value of triplicate GC-FID analysis); arbitrary units were used (*: compound identified by GC-MS only).
[c] Standard deviation (SD) of triple determinations.

enzymic reduction of methyl ketones to secondary alcohols will also occur [7, 12].

One of the most important ketone flavour compounds is diacetyl (2,3-butanedione), which has a buttery, nut-like flavour [46]. Diacetyl is formed in dairy products from citrate by *L. lactis* subsp *lactis* var *diacetylactis* and *Leuconostoc* species. We detected diacetyl in the WSF of all cheeses investigated. High levels were found in the WSF of low-fat Gouda 20$^+$ cheese and in Maasdam cheese. The reduction of diacetyl leads to acetoin, a compound with a woody and mildew aroma [33]. Further reduction of acetoin results in the formation of butanone [1, 44]. Both acetoin and butanone were present in the WSFs of the cheeses. However, their exact sensory contribution remains unclear.

Acetophenone, which has a floral odour [21, 39], was present in trace amounts in the WSF of the cheeses. The compound is believed to originate from phenylalanine via an enzymic process [10, 39]. Non-enzymic formation of acetophenone has also been reported [21].

3.5 Sulphur and miscellaneous compounds

Of special interest for cheese flavour formation are decomposition products of sulphur-containing amino acids (Table 5). The relevance of these, often volatile, sulphur compounds has been known for years [1, 23, 30, 44] and their presence is reported in various cheese types, e.g. Parmesan [7], Cheddar [44], mould-ripened cheeses [19, 20], Limburger cheese [39] and Allgäuer Emmentaler cheese as well as Swiss Emmental cheese [11, 40]. Decomposition of sulphur amino acids during cheese ripening produces volatile sulphur com-

Table 5. Sulphur compounds and miscellaneous components in the water-soluble fraction of eight cheese types[a].

Miscellaneous	Cheese type															
	Gouda 20$^+$		Gouda		Proosdij		Gruyère		Maasdam		Edam		Parmesan		Cheddar	
	Area[b]	SD(%)[c]	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)	Area	SD(%)
Dimethyl-disulphide	*		*		*		*		*		*		*		*	
Dimethyl-trisulphide	*		*		*		*		*		*		*		*	
Dimethyl-benzene	*		*		*		*		*		*		*		*	
Ethyl-benzene	*		*		*		*		*		*		*		*	
Indole							*									
Limonene	106	17	126	27	2617	20	1368	19	1370	10	208	20	1230	29	1535	22
Toluene	*		*		*		*		*		*		*		*	

[a] Tentative identification by spectrum interpretation and by comparison of the spectra with bibliographic data.
[b] Peak area (mean value of triplicate GC-FID analysis); arbitrary units were used (*: compound identified by GC-MS only).
[c] Standard deviation (SD) of triple determinations.

pounds such as hydrogen sulphide and methanethiol. Oxidative reactions can convert the latter to dimethyldisulphide (DMDS) and dimethyltrisulphide (DMTS) [39]. We detected DMDS and DMTS in the WSF of all the cheeses investigated. It was, however, not possible to detect more volatile sulphur compounds, such as hydrogen sulphide and methanethiol. Their absence from the headspace chromatograms was probably caused by poor adsorption to the trapping material after purging [3, 24]. Both DMDS and DMTS are considered to be very important for cheese flavour and especially the odour of DMTS has been described as "overripened-cheese-like" [15]. The importance of other sulphur compounds, e.g. methylthioesters in Limburger cheese, has also been recognised [39]. The formation of methanethiol, the precursor of DMDS, DMTS and probably also of methylthioesters, from methionine may occur via enzymic [2, 29] and non-enzymic [21, 30] pathways.

Besides sulphur compounds, indole and limonene, both organoleptically important, were detected in the WSF of cheeses (Table 5). Indole, considered to be a degradation product of tryptophan, was found only in the WSF of Gruyère cheese. This compound, which has a stable/musty odour, has also been found in French and Spanish cheeses [16, 33]. The monoterpene limonene, which was present at the highest concentration in WSF of Proosdij cheese, has a characteristic odour of citrus fruit [46].

4 Concluding remarks

From the foregoing, it can be assumed that there is not a single compound or class of compounds which is responsible for the full flavour of cheese. The flavour is indisputably due to the presence of numerous components; volatile products of fatty acid and amino acid conversion seem to be particularly important. The breakdown of fatty acids and amino acids is probably governed primarily by enzymic processes, and the starter organisms are a major source of the enzymes involved. Differences in flavour between types of cheese are evoked by the use of distinctive starters during cheese production. However, certain non-starter organisms present in cheeses, e.g. moulds and bacterial surface flora as well as the indigenous flora of raw milk, may also contribute considerably to the formation of flavour compounds. The enzyme activities should be well balanced to avoid excessive production of flavour compounds and thus off-flavour formation. Apart from enzymes, a proper availability of their substrates, produced by breakdown of proteins and fats, is a prerequisite for sufficient formation of flavour components in cheese.

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Chapter 4

Development of cheese flavour from peptides and amino acids by cell-free extracts of *Lactococcus lactis* subsp. *cremoris* B78 in a model system

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Development of cheese flavour from peptides and amino acids by cell-free extracts of *Lactococcus lactis* subsp. *cremoris* B78 in a model system

Summary

The formation of a cheese-like flavour from amino acids and peptides, by the action of enzymes from *Lactococcus lactis* subsp. *cremoris* B78, a Gouda cheese starter organism, was investigated. Cell-free extract (CFE) was obtained from the organism after ultrasonic disruption of cells and subsequent removal of cell debris by centrifugation. The CFE was ultrafiltered to remove low-molecular-mass compounds (<500 Da). The retentate (CFE>500) was used as an enzyme source in incubation experiments with various mixtures of amino acids in water and with peptide solutions. These peptide solutions contained the α_{s1}-casein peptides α_{s1}-CN(f1-23) and α_{s1}-CN(f24-199) or a mixture of peptides of molecular weight 500-5000 Da, isolated from Gouda cheese. The solutions were incubated aseptically for one week at 25 °C. Sensory analysis showed that a cheese-like flavour developed in amino acid mixtures containing free methionine or in mixtures of peptides containing methionine. In the latter case, methionine was released from the peptides by the action of proteolytic enzymes in the CFE>500. The formation of volatile sulphur compounds from methionine could be demonstrated by gas chromatography-mass spectrometry. The results indicated that non-proteolytic enzymes from mesophilic lactococci are important for flavour formation in cheese.

1 Introduction

Maturation of cheese is accompanied by comprehensive proteolysis [8, 35]. The extent of proteolysis is closely related to the formation of cheese flavour and texture, but also to off-flavour formation [9, 37]. The process of proteolysis in ripening cheese has been investigated extensively. It comprises the action of proteolytic enzymes, such as residual chymosin, on intact casein in the cheese curd (primary phase of ripening) and the further breakdown of proteins and polypeptides to small peptides and amino acids by the action of proteinases and peptidases from starter bacteria (secondary phase of ripening) [29, 31, 39].

Products of proteolysis, i.e. small peptides and amino acids, are found in the water-soluble fraction (WSF) of cheese. Because of the considerable contribution of the WSF to the intensity of cheese flavour, this fraction has been studied extensively. It contains both non-volatile and volatile compounds [1, 8, 11]. Fractionation studies of the WSF from several types of cheese showed that cheese flavour components are of low molecular mass (<500 Da). These compounds in WSF are mainly free amino acids and

small peptides [8, 11, 25, 35]. Their contribution to the basic flavour of cheese (e.g. savoury, brothy and bitter) is probably considerable [11]. However, since it is unlikely that the flavour of cheeses such as Cheddar and Gouda results from peptides and amino acids exclusively, other ripening products seem essential for the actual cheese flavours. These include degradation products of free amino acids and free fatty acids, formed via enzymic and/or chemical pathways (third phase of ripening) [9, 34, 38, 39].

Formerly, non-enzymic amino acid degradation has been suggested as a source of sulphur-containing flavour compounds in Cheddar [15, 24] but also in Swiss-type cheese [16]. The degradation of amino acids may also take place via the action of enzymes such as deaminases, decarboxylases, transaminases and enzymes converting amino acid side chains [7, 17]. Microorganisms present in the cheese are the source of these enzymes. Especially organisms involved in surface ripening of cheese such as coryneform bacteria (e.g. *Brevibacterium linens*) and moulds (e.g. *Penicillium roqueforti*), but also starter bacteria like lactobacilli, appear to be capable of producing (volatile) products of amino acid catabolism (e.g. alcohols, aldehydes, sulphur-compounds). Much attention has been paid to research on the mechanisms involved in the catabolism of amino acids by these organisms [5, 20, 23, 32, 33]. However, concerning the significance of amino acid degradation by mesophilic starter bacteria (e.g. *Lactococcus* species) for flavour formation in cheeses, hardly any information is available.

The present paper describes studies on the degradation of peptides and amino acids in a model system, and subsequent flavour formation, by a *Lactococcus lactis* subsp. *cremoris* strain isolated from Gouda cheese starter.

2 Materials and methods

2.1 Chemicals

Free L-amino acids were obtained as Sigma-grade from Sigma Chemical Company, St. Louis, MO, USA. All other reagents were of analytical grade.

2.2 Bacteria and growth conditions

Lactococcus lactis subsp. *cremoris* B78 (NIZO collection, number B358) was maintained and grown in pasteurized milk (30 min, 100 °C), reconstituted from spray-dried, non-fat milk powder (10 % w/v). The strain was isolated from DL-type starter, code Bos. Growth of the cells was started by inoculation with 0.05 % of a 7 h culture and was continued overnight at 20 °C (final pH 5.2 to 5.4, logarithmic phase). The cells were harvested as described earlier [13] and washed twice with an excess of 0.1 M sodium phophate buffer (pH 6.8). The washed cells were resuspended to an OD_{650} of approximately 40 (Uvikon 810, Kontron, Switzerland) in distilled water. The suspension was stored overnight at –20 °C until further use.

2.3 Preparation of cell-free extracts (CFE)

The –20 °C cell suspension was defrosted at 25 °C and subsequently the cells were disrupted ultrasonically at 0 °C (Heat Systems Sonicator XL 2020 NY, USA). Judged by the release of the intracellular enzyme lactate dehydrogenase (LDH) [2] this treatment appeared to be effective for lysis of the cells.

The treated suspension was centrifuged (30 min, 12000 *g*, 4 °C) to remove intact bacteria and cell debris, and the supernatant (CFE), which contained the soluble proteins, was collected. The CFE was ultrafiltered to remove low-molecular-mass components, which could interfere with flavour assessment, and also to avoid the possible contribution of these low-molecular-mass compounds to flavour formation in subsequent incubation experiments. Ultrafiltration (UF) was performed at 4 °C in a stirred-cell type ultrafiltration module (Amicon Corporation, MA, USA), operated under a nitrogen pressure of 300 kPa using Amicon Diaflo membranes YC05 (500 Da molecular mass cut-off, MMCO). The retentate (CFE>500), repeatedly washed with distilled water and refiltered, contained the CFE protein material. Measurement of the LDH activity of the CFE>500 fraction showed no loss of activity compared with total CFE. The permeate (CFE<500) was devoid of LDH activity. Both CFE>500 and CFE<500 were stored on ice until further use.

2.4 Incubation experiments

The production of compounds that contribute to cheese flavour was studied in a series of incubation experiments. As a rule, 4 ml total CFE or CFE>500 was mixed with an equal amount of substrate solution. For certain purposes (e.g. gas chromatography), 10 ml of each were used. The substrate solution consisted of amino acids or peptides in distilled water (see also sections 2.4.1 and 2.4.2). Blank incubations were performed with distilled water as substitute for total CFE, CFE>500 or substrate solution. Heat-treated CFE (10 min 95 °C, followed by centrifugation at 25000 *g* for 30 min) was, after removal of low-molecular-mass components by 500 Da MMCO UF, also used for this purpose. Each incubation was performed at least in duplicate.

2.4.1 Incubation of total CFE and CFE>500 with peptides. The peptide-containing WSF 500<UF<5000 (25 mg peptide/ml) obtained from 6 month old Gouda cheese [11] and the α_{s1}-casein peptides α_{s1}-CN(f1-23) (10 mg/ml in distilled water) and α_{s1}-CN(f24-199) (15 mg/ml in distilled water) [12] were used as substrates. WSF 500<UF<5000 contained peptides with an approximate molecular mass between 500 and 5000 Da.

After addition of 0.1 % NaCl (w/v) (see also section 2.5.3), the pH was adjusted to 5.4 with dilute HCl or NaOH. This pH was similar to that in ripened cheese. Incubations were performed in 50 ml screw-capped bottles, which were heat-sterilized prior to use.

The incubation mixtures were filter-sterilized (Millex-GS, 0.22 µm, Millipore, Molsheim, France) and aseptically transferred to the incubation bottles. The bottles were

capped and then incubated for 7 days in a water bath shaker at 25 °C in the dark. Afterwards the mixtures were stored at −20 °C until further investigation.

2.4.2 Incubation of CFE>500 with amino acids. During incubations with free amino acids, the initial concentration per amino acid in the reaction mixture was 2 mM, unless stated otherwise. This concentration was chosen because it equals the average concentration of free amino acids in the WSF of six month old Gouda cheese (total free amino acid amount approximately 5 mg/ml) [11] which acted as reference fraction in taste trials (see section 2.5.3). The incubation conditions and the handling of the mixtures after incubation were the same as those mentioned in section 2.4.1.

2.5 Analysis

2.5.1 High-Performance Liquid Chromatography (HPLC) and amino acid analysis. The HPLC method used has been described before [11]. Analysis of free amino acids was performed on a 4151 Alpha Plus amino acid analyser (Pharmacia LKB, Uppsala, Sweden). Total amino acids were determined after hydrolysis of the samples with 6 M HCl in evacuated tubes at 110 °C for 24 hours.

2.5.2 Gas Chromatography-Mass Spectrometry (GC-MS). For the identification of volatile components in the incubation mixtures, purge and trap thermal desorption cold-trap (TDCT) gas-chromatography-mass spectrometry was used. A 15 ml quantity of the incubation mixture was purged with 150 ml/min helium for 30 min at room temperature. Volatile components were collected on a trap containing Carbosieve SIII and Carbotrap. The trapped components were transferred onto the column of a gas chromatograph (Hewlett Packard HP 5890A), using the Chrompack PTI injector in the TDCT mode, by heating the trap for 10 min (250 °C). A narrow injection band of the components was achieved by cryofocussing (−100 °C).

The gas chromatograph was equipped with a 60 m × 0.32 mm i.d. fused silica capillary DB1 column (film thickness 1.0 µm) and the injector-block temperature was 200 °C. The carrier gas was helium (column pressure 80 kPa). The oven-temperature programme was: 1 min at 40 °C, 10 °C/min to 50 °C, 1 min at 50 °C, 1 °C/min to 60 °C, 10 °C/min to 250 °C and 15 min at 250 °C. The GC column was directly connected to the ion source (temperature 200 °C) of a VG 12-250 quadrupole mass spectrometer (temperature of interface line: 210 °C). The MS was operating in the scan mode from 25 to 500 Da at 1 scan/s. Ionisation was by electron impact at 70 eV. Structures were assigned by spectrum interpretation and comparison of the spectra with bibliographic data.

2.5.3 Sensory analysis. The incubation mixtures stored at −20 °C were defrosted and subsequently ultrafiltered (500 Da MMCO, for the procedure see section 2.3). The permeate fractions were used for taste evaluation. Preceding taste trials showed that

flavour components completely moved into the permeate fraction during the UF treatment. Cheese flavour was never observed in the UF retentates of the incubation mixtures. Consequently these retentates were not investigated further. The permeates were freeze-dried and dissolved in half the original volume of distilled water. Preliminary experiments revealed that although freeze-drying resulted in some loss of flavour, a fair assessment of the flavour of each sample was feasible.

The various samples were analysed by a panel of five or six experienced tasters. During round-table discussion sessions the panellists tasted 400 µl aliquots, administered directly onto the tongue. The overall flavour quality (scale ranging from 3 [very poor] to 8 [very good]) and the cheese flavour intensity (scale ranging from 0 [none] to 4 [very strong]) were scored. Overall flavour quality means the general appreciation of the sample, i.e. not focussed on cheese flavour exclusively.

The trials were performed 'blind' and the taste of the samples was assessed taking fraction UF<500 from 6 month old Gouda cheese [11] as reference, which by definition had an overall flavour quality of 6.5 and a cheese flavour intensity of 1.0. This fraction contained only small (<500 Da) water-soluble components. The concentration of NaCl in the reference fraction was equal to the concentration of NaCl in the samples. The averages of the individual scores are presented.

3 Results

3.1 Incubation of total CFE and CFE>500 with peptides

The formation of cheese-like flavours from casein-derived peptides by CFE from *Lactococcus lactis* subsp. *cremoris* B78 was investigated during a series of *in-vitro* incubation experiments. As substrate a fairly heterogeneous peptide fraction from Gouda cheese (WSF 500<UF<5000) was used, and in addition two α_{s1}-casein fragments (α_{s1}-CN(f1-23) and α_{s1}-CN(f24-199)).

In Table 1 the results of taste trials with incubation mixtures consisting of CFE and the Gouda cheese peptide fraction are shown. The formation of a cheese-like flavour occurred and both taste and odour were comparable with those of the reference from 6-month-old Gouda cheese. CFE>500, i.e. the fraction without low-molecular-mass components, was as effective as total CFE in flavour formation. Therefore, only CFE>500 was used during subsequent experiments. The use of CFE<500 never led to cheese flavour formation.

Reversed-phase HPLC profiles showed that the peptide fraction (WSF 500<UF<5000) from Gouda cheese contained mainly peptides with longer retention times (Figure 1, trace 1). During incubation with CFE>500, these hydrophobic peptides were almost totally converted, by the action of proteolytic enzymes in CFE>500, to components with lower retention times such as very small peptides and free amino acids (Figure 1, trace 2). The profile became similar to that of the UF<500 fraction from Gouda cheese [11] shown in Figure 1, trace 3. This UF<500 fraction contained

approximately 80 % free amino acids. The peaks at 6.7 min, 11.7 min and 25.2 min were due to tyrosine, phenylalanine and tryptophan, respectively.

Table 1. Mean flavour scores of a Gouda cheese peptide mixture[a] (500<UF<5000) incubated with total cell-free extract (CFE), CFE>500 and CFE<500, all obtained from *Lactococcus lactis* subsp. *cremoris* B78.

Incubation	Overall flavour quality[b]	Cheese flavour intensity[c]
CFE + peptide mixture	6.5	1.0
CFE + water	5.0	0
water + peptide mixture	5.0	0.1
CFE>500 + peptide mixture	6.3	1.1
CFE>500 + water	4.9	0.1
CFE<500 + peptide mixture	6.0	0.1

[a] 12.5 mg peptide/ml incubation mixture
[b] scale 3 to 8
[c] scale 0 to 4

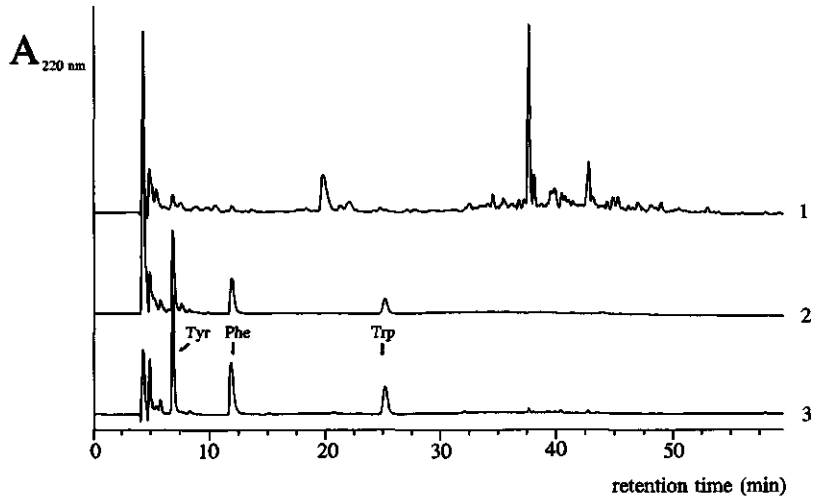

Figure 1. Reversed-phase high-performance liquid chromatography results of the incubation of a peptide fraction from Gouda cheese (500<UF<5000) with the cell-free extract fraction CFE>500 of *Lactococcus lactis* subsp. *cremoris* B78. 1, 500<UF<5000 + water; 2, 500<UF<5000 + CFE>500; 3, UF<500 from Gouda cheese (reference).

Amino acid analysis revealed that 98.5 % of the cheese peptide fraction WSF 500<UF<5000 was converted to free amino acids by the action of CFE>500. In Figure 2 the amounts of individual free amino acids formed during incubation are displayed (open bars). Glutamic acid, proline and lysine predominated among the amino acids liberated.

A certain degree of (auto)proteolysis was also observed in the control incubation mixture with CFE>500 but without peptide substrate (CFE>500+water, Figure 2, filled bars). Due to this process, overall enzyme activity was expected to diminish gradually during incubation.

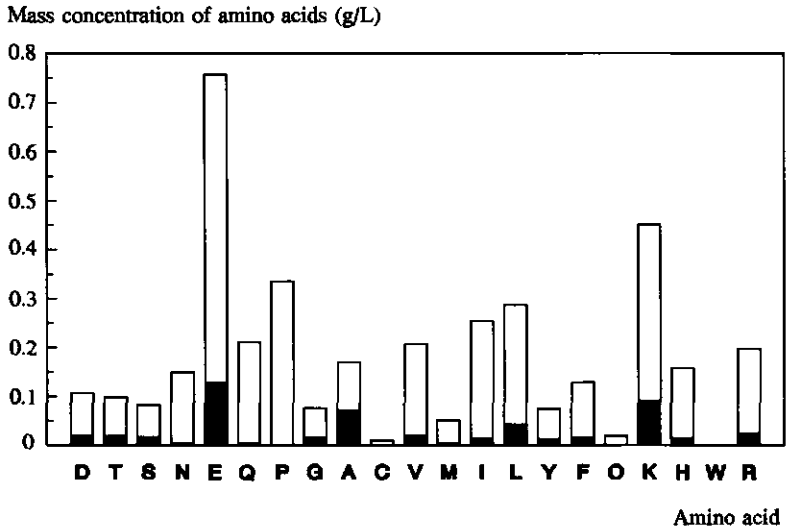

Figure 2. Free amino acids formed during incubation of Gouda cheese peptide fraction 500<UF<5000 with cell-free extract fraction CFE>500 of *Lactococcus lactis* subsp. *cremoris* B78. Filled bars: free amino acids formed during blank incubation (CFE>500 + water); open bars: free amino acids formed during incubation of CFE>500 + peptide fraction 500<UF<5000. One-letter codes were used for the notation of amino acids [18]; O, ornithine.

The incubation of individual peptide substrates α_{s1}-CN(f1-23) and α_{s1}-CN(f24-199) with CFE>500 both resulted in breakdown of the peptides. Analysis of the incubation mixtures by reversed-phase HPLC revealed the presence of proteolytic cleavage products. The peaks due to tyrosine, phenylalanine and tryptophan, partly due to autoproteolysis in the CFE fraction, are marked (Figure 3, traces 1 and 2). Other amino acids generated did not exhibit absorbance at 220 nm. With α_{s1}-CN(f1-23) no appreciable cheese flavour formation occurred (Table 2), although the peptide was fully

degraded (Figure 3, trace 2). The peptide α_{s1}-CN(f24-199) was almost completely degraded during the incubation with CFE>500 (Figure 3, trace 1). A cheese-like flavour

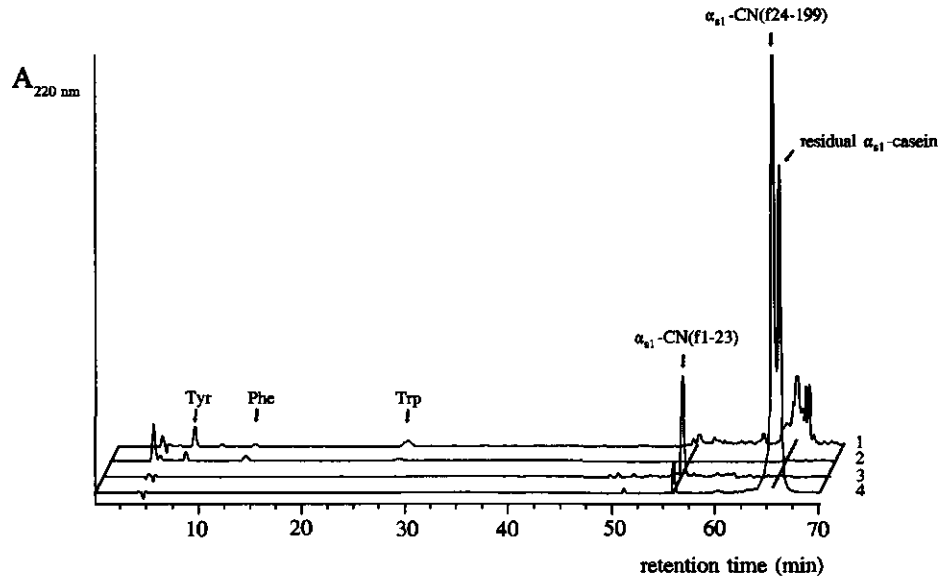

Figure 3. Reversed-phase high-performance liquid chromatography results of the incubation of α_{s1}-casein peptides with cell-free extract fraction CFE>500 of *Lactococcus lactis* subsp. *cremoris* B78. 1, α_{s1}-CN(f24-199) + CFE>500; 2, α_{s1}-CN(f1-23) + CFE>500; 3, α_{s1}-CN(f1-23); 4, α_{s1}-CN(f24-199).

developed and both taste and odour were comparable with those of the reference (Table 2). The incubation of a higher concentration (40 mg/ml) of the α_{s1}-CN(f24-199) substrate solution with CFE>500 resulted in the formation of an intense cabbage-like flavour, which was also denoted as Limburger cheese-like by some of the graders. The important contribution of volatile sulphur compounds to Limburger cheese aroma has been known for many years [30].

3.2 Incubations of CFE>500 with amino acid mixtures

The taste-trial results presented in the previous section suggested that some of the constituent amino acids from the peptides examined could play an important role in flavour formation. To further test the direct contribution of individual amino acids or groups of amino acids to a cheese-like flavour, a series of experiments was carried out. In a preliminary experiment a mixture of amino acids in water was prepared in which

Table 2. Mean flavour scores of α_{s1}-casein peptides with cell-free extract fraction CFE>500 from *Lactococcus lactis* subsp. *cremoris* B78.

Incubation	Overall flavour quality[a]	Cheese flavour intensity[b]
CFE>500 + α_{s1}-CN(f1-23)[c]	5.6	0.3
water + α_{s1}-CN(f1-23)[c]	4.7	0.2
CFE>500 + α_{s1}-CN(f24-199)[d]	5.8	0.9
water + α_{s1}-CN(f24-199)[d]	4.8	0.1

[a] scale 3 to 8
[b] scale 0 to 4
[c] 5 mg α_{s1}-CN(f1-23)/ml incubation mixture
[d] 7.5 mg α_{s1}-CN(f24-199)/ml incubation mixture

the concentration of each amino acid was equal to its concentration in a UF<500 fraction from Gouda cheese investigated in an earlier study [11]. The cheese flavour of this mixture of intact amino acids was negligible as compared with the cheese flavour of the fraction UF<500 from Gouda cheese. Therefore, incubation experiments were conducted in which amino acids were used as substrates for (non-proteolytic) enzymes from *Lactococcus lactis* subsp. *cremoris* B78. The composition of the amino acid mixtures used and the results of the experiments are shown in Tables 3 and 4. The only mixture in which an appreciable cheese-like flavour was generated contained amino acids as well as CFE>500 (Table 3, mixture 1). Omission either of enzyme-containing CFE>500 (Table 3, mixture 2 and 4) or of amino acid substrate (Table 3, mixture 3) drastically reduced cheese flavour formation. The extent of free amino acid formation due to proteolysis of protein material in the CFE>500 was comparable with that mentioned in section 3.1 for CFE>500 + water. These amounts were too small to pro-

Table 3. Mean flavour scores of a total amino acid mixture[a] incubated with cell-free extract fractions CFE<500 and CFE>500 from *Lactococcus lactis* subsp. *cremoris* B78.

Incubation	Overall flavour quality[b]	Cheese flavour intensity[c]
1. total amino acid mixture + CFE>500	6.2	0.9
2. total amino acid mixture + water	5.2	0.3
3. water + CFE>500	5.0	0.2
4. total amino acid mixture + CFE<500	5.6	0.2
5. water + CFE<500	4.7	0

[a] composition: Ala, Arg, Asn, Asp, Cys, Gaba, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Orn, Phe, Pro, Ser, Thr, Trp, Tyr, Val. The concentration per amino acid in the incubation mixture was 2 mM.
[b] scale 3 to 8
[c] scale 0 to 4

Table 4. Mean flavour scores of incubations of amino acids with cell-free extract fraction CFE>500 from *Lactococcus lactis* subsp. *cremoris* B78[a].

Amino acid	Incubation mixtures									
	1	2	3	4	5	6	7	8	9	10
Ala	x[b]	x	x	x	x	x	x	x	x	
Leu	x	x	x	x	x	x	x	x	x	
Ile	x	x	x	x	x	x	x	x	x	
Val	x	x	x	x	x	x	x	x	x	
Gly	x	x	x	x	x	x	x	x	x	
Pro	x	x	x	x	x	x	x	x	x	
Gaba	x	x	x	x	x	x	x	x	x	
His		x								
Arg		x								
Lys		x								
Orn		x								
Trp			x							
Tyr			x							
Phe			x							
Glu				x						
Gln				x						
Asp				x						
Asn				x						
Ser					x	x				
Thr					x		x			
Cys					x			x		
Met					x				x	x
Overall flavour quality[c]	5.3	5.6	5.2	5.3	6.2	6.0	5.7	5.8	6.3	6.4
Cheese flavour intensity[d]	0.2	0.2	0.1	0.2	0.8	0.4	0.5	0.4	0.9	1.0

[a] Blank incubations with amino acids only revealed no cheese flavour formation (see table 3).
[b] x = amino acid is present in the incubation mixture; concentration per amino acid in the mixtures was 2 mM.
[c] scale 3 to 8
[d] scale 0 to 4

mote substantial cheese flavour formation (Table 3, mixture 3). Since the results presented support the concept that enzymic conversion of amino acids is essential for cheese flavour formation, further incubation experiments were conducted to determine the contribution of individual amino acids (Table 4). A base substrate mixture consti-

tuted of seven amino acids, to which one or more amino acids had been added, was used for this purpose. As can be seen in Table 4, incubation of the mixtures with CFE>500 resulted in a noticable cheese flavour when a combination of serine, threonine, cysteine and methionine was added to the seven base amino acids (Table 4, incubation 5). Absence of certain amino acids from the added combination of amino acids reduced cheese flavour formation (Table 4, incubations 6 to 8). An important factor for formation of a cheese-like flavour in the mixtures appeared to be the presence of methionine (Table 4, incubation 9). This is further supported by the fact that incubation of methionine solely with CFE>500 of *Lactococcus lactis* subsp. *cremoris* B78 resulted in the development of a flavour which was comparable both in overall flavour quality and in cheese flavour intensity with the reference (Table 4, incubation 10). The use of heat-treated CFE in incubation experiments with methionine did not result in formation of a cheese-like flavour. Moreover, no cheese-like flavour developed during control incubations (CFE>500 + water), in which formation of free methionine (due to autoproteolysis) is absent (Figure 2, filled bars).

After incubation of mixtures of methionine with CFE>500 GC-MS analysis revealed the formation of the following volatile sulphur compounds:

hydrogen sulphide	H_2S
carbon disulphide	CS_2
methanethiol	CH_3SH
dimethylsulphide	CH_3SCH_3
dimethyldisulphide	CH_3SSCH_3
dimethyltrisulphide	CH_3SSSCH_3

Due to the MS identification technique applied, we were only able to determine the relative amounts of compounds produced (Figure 4). Comparison of these relative amounts was possible for homologous compounds. The MS analysis revealed that the levels of methanethiol, dimethyltrisulphide and particularly dimethyldisulphide were elevated during incubation of methionine with CFE>500 (Figure 4). The formation, to some extent, of volatile sulphur compounds in the methionine + water incubation mixture was probably due to chemical conversion of methionine.

Apart from sulphur compounds other volatiles produced during incubation of CFE>500 with amino acids were identified. An example is 3-methylbutanal, which probably originated from leucine [26].

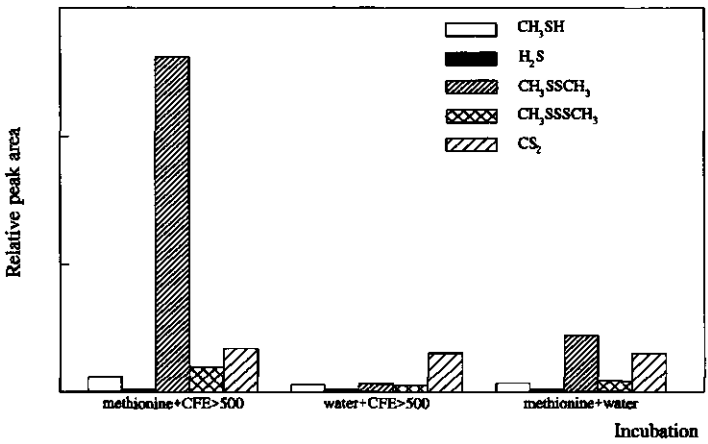

Figure 4. Relative amounts of sulphur compounds formed during incubation of methionine with cell-free extract fraction CFE>500 of *Lactococcus lactis* subsp. *cremoris* B78.

4 Discussion

The flavour profile of mature cheese varieties is extremely complex. The principal flavour constituents are in the WSF of cheese [1, 11, 19]. The levels of non-volatile, peptide and amino acid components in this fraction increase with ripening time, and these components have therefore been associated with flavour development [8, 28]. However for the actual cheese flavour, the role of peptides and amino acids, produced by the action of chymosin, plasmin and proteolytic enzymes of the starter organisms, is probably limited [11, 29, 39]. Modification of amino acids, but also of fat and, in some cheese types, lactic acid [7], to volatile products seems essential.

The formation of volatiles during cheese ripening is prominent and has been extensively examined [3, 4, 6, 14, 38]. Studies on the actual role of the WSF components of interest are usually difficult because of the complicated procedures necessary to isolate and examine individual components. The fractions obtained are often less suitable for sensory assessment.

In the present study the formation of a Gouda cheese-like flavour, by the action of CFE from *Lactococcus lactis* subsp. *cremoris* B78 on various peptide and amino acid substrates, was examined. Total CFE and the CFE>500 fraction of this microorganism exhibited strong proteolytic activity towards peptide fractions. This activity resulted in breakdown of, for instance, the peptide fraction 500<UF<5000 from Gouda cheese, which itself was almost completely devoid of flavour and was degraded essentially to free amino acids. Degradation products of these amino acids were probably also formed.

This was accompanied by formation of a cheese-like flavour. The flavour intensity equalled that of a reference fraction from Gouda cheese (UF<500).

The α_{s1}-casein peptides α_{s1}-CN(f1-23) and α_{s1}-CN(f24-199) were, for the greater part, also degraded to free amino acids by action of enzymes in CFE. The direct contribution of the peptides generated and of the remaining α_{s1}-CN(f24-199) to the cheese flavour is thought to be small [11, 29]. Further modification of amino acids seems essential. This view was supported by the incubation experiments with free amino acids as substrate. However, the presence of CFE was a prerequisite for the formation of a cheese-like flavour. In the case of α_{s1}-CN(f24-199), the distinct flavour formation presumably resulted from further enzymic breakdown of certain key amino acids (e.g. methionine, serine and threonine) which were absent in α_{s1}-CN(f1-23). With this peptide as a substrate no significant cheese flavour developed (Table 2).

The existence of a relationship between cheese flavour and the content of amino acids has been assumed for a long time [27, 36]. Amino acids are end products of proteolysis, which accumulate during cheese ripening. Differences between various cheese types in the breakdown of amino acids will contribute to the formation of distinct flavours. Our incubation experiments revealed that methionine is an important precursor for the formation of a cheese-like flavour with CFE>500 from *Lactococcus lactis* subsp. *cremoris* B78. The fact that heat-treatment of the CFE fraction abolished its ability to generate a cheese-like flavour, on incubation with methionine, points to an enzymic character of the amino acid conversion, although chemical conversion, to some extent, could not be ruled out (see Figure 4). The simultaneous formation of volatile products of methionine also suggested the importance of methionine breakdown. To a lesser extent the amino acids threonine, serine and cysteine contributed to the formation of a cheese-like flavour. Breakdown of other amino acids (e.g. leucine) was observed as well. The formation of sulphur compounds from methionine by *Brevibacterium linens* and *Pseudomonas* species has been reported [21, 22].

The importance of certain sulphur compounds for cheese flavour has been recognised before. Dimethyldisulphide and dimethyltrisulphide were, by some researchers [10, 20], reported to contribute substantially to cheese flavour. Others [38], however, questioned their significance for cheese flavour. At higher concentrations of these sulphur compounds, a cabbage-like flavour develops, as was the case when we incubated a fairly high amount of, methionine containing, α_{s1}-CN(f24-199) with CFE>500. The direct contribution of the sulphur compounds detected in the incubation mixtures to the observed Gouda cheese-like flavour therefore remains unclear. During incubations a low redox potential, as exists in the cheese interior [29], was probably not attained because experiments were performed under aerobic conditions. In cheese, oxidation processes, such as conversion of methanethiol to dimethyldisulphide, presumably proceed at a relatively slow rate enabling other methanethiol conversions. Recent experiments performed at our laboratory indicated that incubation of methionine with CFE>500 under reduced oxygen pressure inhibited the formation of dimethyldisulphide. Instead, formation of another volatile sulphur compound was observed (results not shown).

In summary, we demonstrated that non-proteolytic enzymes of mesophilic lactococci, present in hard-type cheeses such as Gouda, are probably very important for development of a genuine cheese flavour. In other, non-Gouda, types of cheese a variety of starter and non-starter organisms are present. The breakdown of amino acids by enzymes of these organisms might also be related to the formation of characteristic cheese flavours. Even a low metabolic activity could play a significant role in the production of cheese flavour compounds from methionine and other amino acids because of the duration of cheese ripening and the often low taste or aroma thresholds of the products [9].

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Chapter 5

Purification and characterization of cystathionine β-lyase from *Lactococcus lactis* subsp. *cremoris* B78 and its possible role in flavour development in cheese

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Purification and characterization of cystathionine β-lyase from *Lactococcus lactis* subsp. *cremoris* B78 and its possible role in flavour development in cheese

Summary

An enzyme that degrades sulfur-containing amino acids was purified from *Lactococcus lactis* subsp. *cremoris* B78; this strain was isolated from a mixed-strain, mesophilic starter culture used for the production of Gouda cheese. The enzyme has features of a cystathionine β-lyase (EC 4.4.1.8), a pyridoxal-5′-phosphate-dependent enzyme involved in the biosynthesis of methionine and catalyzing an α,β-elimination reaction. It is able to catalyze an α,γ-elimination reaction as well, which in the case of methionine, results in the production of methanethiol, a putative precursor of important flavour compounds in cheese. The native enzyme has a molecular mass of approximately 130 to 165 kDa and consists of four identical subunits of 35 to 40 kDa. The enzyme is relatively thermostable and has a pH optimum for activity around 8.0; it is still active under cheese-ripening conditions, viz., pH 5.2 to 5.4 and 4% (w/v) NaCl. A possible essential role of the enzyme in flavour development in cheese is suggested.

1 Introduction

Proteolysis in Gouda cheese is essential for the ultimate generation of the typical cheese flavour. Chymosin, the main constituent of rennet, is primarily responsible for initial cleavage of caseins in cheese [43] while the action of the lactococcal cell-envelope proteinase is essential for an efficient secondary proteolysis, leading to the formation of small peptides and amino acids [17, 18], and for a normal flavour development [37].

The typical flavour of Gouda cheese and of other cheese types is recovered in the water- soluble fraction of the cheeses [12]. The results of fractionation experiments has shown that low-molecular-mass components (<500 Da) are responsible for these typical cheese flavours. The water-soluble fraction contains small peptides, amino acids, free fatty acids and possibly degradation products thereof. Although it is known that peptides can taste bitter [28] or delicious [44] and that amino acids can taste sweet, bitter or broth-like [33], the direct contribution of peptides and amino acids to flavour is probably limited to a basic taste. The components responsible for the typical cheese flavour are assumed to be part of the volatile fraction and they may be associated with proteolysis via the production of specific amino acids, which are the precursors of these flavour components. In fact, a Gouda-cheese-like flavour can be generated by incubating methionine with the cell-free extract of *Lactococcus lactis* subsp. *cremoris* strain B78 [13].

The degradation of amino acids may take place via enzymic pathways involving different types of enzymes, which either act as a deaminase, decarboxylase or transaminase, or convert amino acid side chains [24, 26]. Since such enzymes are likely to be located intracellularly, lysis or permeabilization of starter bacteria is a prerequisite for the accessibility of these enzymes to external substrates. In fact, the appearance in a young Gouda cheese of specific degradation products of the action of an intracellular endopeptidase indicates lysis of starter bacteria at an early stage of the cheese-ripening process [2, 17].

As part of our work on the relation between proteolysis and flavour development in Gouda cheese [16, 17, 18], we are studying the enzymic degradation of amino acids by lactococci, which is supposed to be an important step in the production of flavour components. In this paper, the isolation, purification and characterization of an enzyme with features of cystathionine β-lyase, from *L. lactis* subsp. *cremoris,* are described. The enzyme is able to degrade various sulfur-containing amino acids and is active under cheese-ripening conditions.

2 Materials and methods

2.1 Chemicals

All amino acids and amino acid derivatives, pyruvic acid, iodoacetic acid, iodoacetamide, hydroxylamine, DL-penicillamine, phenylhydrazine, DL-cycloserine and semicarbazide were obtained from Sigma Chemicals (St Louis, MO), ethylenediaminetetra-acetic acid (EDTA) from BDH Limited (Poole, UK), carboxymethoxylamine, 3-methyl-2-benzothiazolinonhydrazone hydrochloride hydrate and 4-methylthio-2-oxobutyric acid (KMBA) were obtained from Aldrich-Chemie GmbH & Co. (Steinheim, Germany), 5,5′-dithiobis-(2-nitrobenzoic acid) was obtained from Janssen Chimica (Beerse, Belgium), pyridoxal-5′-phosphate (PLP) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany), N-ethylmaleimide was obtained from Merck (Darmstadt, Germany) and Nisin A was purified from Nisaplin (Nisin A content 2.5% w/w, Aplin & Barrett, trowbridge, UK) [36].

2.2 Organism and growth

Strain B78 was isolated from the mixed-strain, mesophilic starter culture Bos (used in The Netherlands for the production of Gouda-type cheese). This DL-type starter consists of mainly *Lactococcus lactis* subsp. *cremoris* strains, *L. lactis* subsp. *lactis* and the citric acid-fermenting *L. lactis* subsp. *lactis* var. *diacetylactis* (D) and *Leuconostoc* (L) strains. Strain B78 was identified as *L. lactis* subsp. *cremoris*. The organism was grown (overnight at 30°C; final pH 4.6 to 4.8) in milk and subsequently harvested as described by Exterkate [15]. The organism requires methionine for its growth in the methionine assay medium of Difco (Detroit, MI).

2.3 Purification

Washed cells from a 7.5 l overnight culture, suspended in 150 ml of a 20 mM potassium phosphate (KPi) buffer (pH 7.5), containing 1 mM EDTA and 20 μM PLP, were frozen in liquid nitrogen and after thawing the cells were disrupted by sonicating the suspension for 16 periods of 15 s (on ice, thereby keeping the temperature below 10 °C) using the XL2020 sonicator of Heat Systems Ultrasonics (New York, NY). The disrupted cells were centrifuged for 30 min at 30,000 g in a J2-21M centrifuge (Beckman Instruments, Palo Alto, CA) to remove intact cells and cell particles from the crude cell extract. Solid ammonium sulfate was added to the cell-free extract to a final concentration of 35% (w/v). Insoluble material was removed by centrifugation (30,000 g) and additional ammonium sulfate was added to a final concentration of 55% (w/v) to precipitate the enzyme-containing material. After a subsequent centrifugation step the precipitate was collected and dissolved in the KPi-EDTA-PLP buffer described above. This solution was extensively washed and concentrated in the same buffer with a 200 ml ultrafiltration cell (Amicon Corporation, Danvers, MA) equipped with a 30 kDa cut-off Omega membrane (Filtron, Northborough, MA). The retentate was filtered through an 0.22 μm pore-size filter (Millipore Corporation, Bedford, MA) and injected (2 ml fractions) onto a MonoQ HR 5/5 (Pharmacia, Uppsala, Sweden) anion-exchange column, equilibrated with the KPi-EDTA-PLP-buffer. Proteins were eluted at 1 ml min^{-1} with a 0.1 to 0.45 mM NaCl gradient in the same buffer. Fractions containing the enzyme activity were pooled, concentrated and filtered as described above and subsequently injected (500 μl fractions) onto a Superose 12 HR 10/30 (Pharmacia) gel-filtration column, equilibrated with KPi-EDTA-PLP buffer. After elution at 0.4 ml min^{-1} the fractions containing the enzyme activity were pooled. The column was calibrated with the following marker proteins: ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa (plus dimer 136 kDa)), ovalbumin (45 kDa), β-lactoglobulin (36 kDa dimer), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa). All were obtained from Pharmacia (except β-lactoglobulin which was purified from whey). All steps were carried out at 0 to 4 °C, and enzyme fractions were stored at -20 °C.

2.4 Enzyme assays

In order to trace the enzyme activity during the different purification steps and to characterize the enzyme, we routinely used the method described by Esaki and Soda [14] for the determination of keto-acid production (for characterization a protein concentration of 7 μg ml^{-1} in a final volume of 500 μl was used) and the method described by Uren [42] for the determination of thiol formation (for characterization a protein concentration of 3.5 μg ml^{-1} in a final volume of 1 ml was used). In both cases cystathionine (5 mM, final concentration) was used as a substrate and the incubation mixtures in 0.1 M KPi-20 μM PLP (pH 8.0) were held at 30 °C for 15 min. For the

specificity studies with the purified enzyme, other amino acids and amino acid derivatives were used as substrate under similar conditions. The influences of inhibitors were established after a 10 min preincubation of the purified enzyme fraction at room temperature with different inhibitors at the indicated concentrations at pH 8.0. To investigate the pH dependence, two buffer systems were used: KPi-Tris-glycine (pH 6.0 to 9.5) and sodium acetate-KPi-Tris (pH 4.5 to 8.0), at a concentration of 0.05 M for each constituent. The temperature sensitivity (stability and optimum) was determined at the pH optimum (pH 8.0). The thermal stability of the enzyme was examined by heating samples in buffer (0.1 M KPi-20 µM PLP, pH 7.0) for 10 min at various temperatures (20 to 70 °C) prior to the assay. Ammonia production was measured by the ammonia determination test of Boehringer (Mannheim, Germany). A Cary 1E UV-VIS spectrophometer (Varian, Victoria, Australia) was used in all cases.

2.5 Identification of reaction products

Substrate degradation was monitored and product identification was performed using an LKB type 451 amino acid analyzer (Pharmacia). Dynamic headspace gas-chromatographic (GC) analyses were performed for identification of the volatile sulfur-containing compounds formed during the degradation of methionine by the action of the purified enzyme. For that purpose a 10-ml sample was purged with helium (150 ml/min, 30 min) at room temperature and the volatile compounds were collected on an adsorption trap packed with Carbotrap (80 mg) and Carbosieve SIII (10 mg). After the sampling procedure the different compounds were desorbed (10 min, 250 °C) and cryofocused (-100 °C) onto the head of the capillary column of the GC using a thermal-desorption-coldtrap injection device (Chrompack, Middelburg, The Netherlands) connected to a Carlo Erba MEGA 5360 GC equipped with a flame-photometric detector (SSD 250) in the sulfur mode (Carlo Erba, Milan, Italy). GC separations were performed with a fused silica capillary column (60 m x 0.32 mm) coated with DB1 (df=1.0 µm) in stationary phase (J&W Scientific, Folsom, CA). During analysis, the oven temperature was held at 40 °C for 1 min, then raised (10 °C min^{-1}) to 50 °C and kept at this temperature for 1 min, raised (2 °C min^{-1}) to 60 °C and then programmed at a rate of 10 °C min^{-1} to 245 °C; this temperature was maintained for 25 min. Identification of sulfur-containing compounds was achieved using retention times of standard compounds. Thin-layer chromatography (TLC) was used to study the possibility of KMBA formation from methionine by the action of the purified enzyme. Samples were withdrawn from an incubation mixture and applied to a cellulose TLC plate (Merck) and developed in butanol/acetone/dimethylamine (40% aqueous solution)/water (40:40:8:20 v/v). Methionine and KMBA were applied as standards. Both could be located by spraying the TLC plate first with 0.1 M potassium bichromate solution in water/acetic acid (1:1 v/v), and then by spraying it with 0.1 M silver nitrate solution in water. Sulfur-containing compounds appeared as yellow derivatives.

2.6 Protein quantitation

Protein concentrations were estimated by the micromethod of Bradford [4] with the Coomassie Protein assay reagent and the instructions of the Pierce Chemical Company (Rockford, Ill) and with crystalline serum albumin (fraction V; BDH, Poole, UK) as the standard.

2.7 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed with a Phast system, a Midget Cell (Pharmacia, Uppsala, Sweden) or a Mini-protean II Cell (Bio-Rad laboratories, Hercules, CA), all according to the respective instruction manuals. Sodium dodecyl sulfate (SDS)-PAGE was carried out with 12.5% polyacrylamide gels with either the Phast system or the Midget cell. Native PAGE was performed using the Mini Protean II cell in combination with 4 to 15% gradient Mini Protean II ready gel (Bio-Rad). The proteins were visualized by Coomassie Brilliant Blue staining or silver staining, with Phastgel blue R and Phastgel silver kit (Pharmacia), respectively. Low- and high-molecular-mass marker proteins (Pharmacia) were used as references.

3 Results

3.1 Purification

Table 1 shows the purification scheme of cystathionine lyase. Most of the methionine-converting activity in the crude cell-free extract appeared to be co-purified with this

Table 1. Purification scheme for the cystathionine lyase activity of *Lactococcus lactis* subsp. *cremoris* B78.

Step	Total protein (mg)	Total activity (U[a])	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Cell-free extract	980	13.2	0.014	1.0	100
Ammonium sulfate precipitation	185	12.2	0.066	4.7	92
Anion-exchange chromatography	8.4	9.3	1.1	76	71
Gel-filtration chromatography	1.7	3.5	2.1	150	21

[a] one unit (U) is defined as 1 μmol thiol generated per minute

lyase (described below). The enzyme was purified 150-fold in a three-step procedure with 21 % recovery. After desalting of the various ammonium sulfate fractions, most of the thiol-generating activity appeared to be present in the 35 to 55% ammonium sulfate (w/v) fraction. With anion-exchange chromatography for the next purification step, all the activity was eluted between 0.35 and 0.38 M NaCl. In the final gel-filtration step, most activity was eluted at 165 kDa; the purest fraction was used for characterization.

The protein was judged to be homogeneous by SDS-PAGE (12.5 % polyacrylamide) stained with Coomassie Brilliant Blue (Figure 1A). Two additional bands, corresponding to low-molecular-mass proteins, could be detected with the more sensitive silver staining (Figure 1B). After separation of the gel-filtration fraction in a native 4 to 15 % gradient polyacrylamide gel, the single band running at an apparent molecular mass of 130 kDa was cut out of the gel, and protein was then extracted with 20 mM KPi (pH 7.5)-20 µM PLP as extraction buffer. This fraction contained both the cystathioninase and methioninase activities and appeared as a single band of 35 to 40 kDa by SDS-PAGE (12.5 % polyacrylamide) followed by silver staining (Figure 1B).

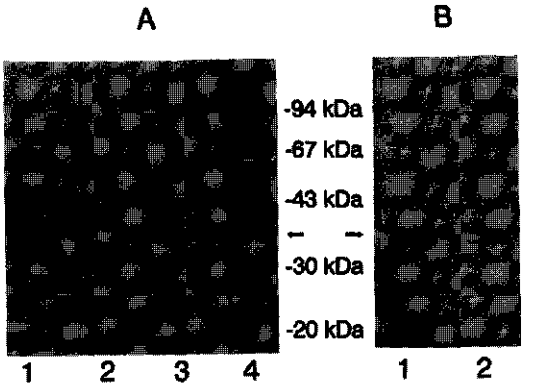

Figure 1. SDS-PAGE (12.5 % polyacrylamide) in combination with a Coomassie Brilliant Blue staining (A) or silver staining (B) showing the different purification steps of the cystathionine lyase activity. (A) lanes 1-4: the 35 to 55 % AS-fraction, the MonoQ-fraction, the Superose 12-fraction, and the Pharmacia low-molecular-mass markers, respectively. (B) lanes 1-2: the Superose 12-fraction and the fraction extracted from the 130 kDa band (4-15 % gradient PAGE), respectively. The position of cystathionine β-lyase is indicated by the arrows.

3.2 Enzyme characterization

The molecular mass of the native enzyme estimated by gel filtration is approximately 165 kDa, which is somewhat higher than that estimated by gradient PAGE (described above). The activity of the purified enzyme was optimal between pH 7.5 and 8.5 with cystathionine as substrate. The activity in the pH range 5.0 to 5.5 was about 10 to 15% of the activity at the optimal pH. The enzyme activity decreased rapidly above pH 8.5. The enzyme was stable up to 60°C over a period of 10 min. The activity declined rapidly above 60°C and the enzyme was completely inactivated by being heated at temperatures above 70°C. There was no significant loss of activity after storage for 20h

Table 2. Substrate specificity of the purified cystathionine lyase.

Substrate[a]	Substrate Concentration (mM)	Relative activity (%)[b]
Lanthionine[c]	5	169
L-Cystathionine	5	100
L-Homoserine	10	96
L-Cystine	5	64
L-Djenkolic acid	5	58
L-Cysteine	10	12
L-Homocysteine	5	4
L-Methionine	10	1

[a] No activity could be measured with the following substrates: L-arginine (5 mM), L-asparagine (5 mM), L-aspartic acid (5 mM), L-glutamic acid (5 mM), L-glutamine (5 mM), L-histidine (5 mM), L-lysine (5 mM), 4-methylthio-2-oxobutyric acid (10 mM) nisin A (1 mg/ml). The last two substrates were measured by thiol determination for enzyme activity.
[b] keto acid determination.
[c] a mixture of D,L- and *meso*-lanthionine.

at 4 °C or 20 °C or after lyophilizing samples containing the enzymic activity; repeated freezing (-20°C) and thawing or storage of the preparations for months at -20°C also had no dramatic effect on the enzyme activity.

In order to demonstrate the cofactor requirement, the purified enzyme, with or without a hydroxylamine treatment (10 mM), was dialyzed against a large volume of 20 mM KPi (pH 7.5) in a Slide-a-lyzer (Pierce Chemical Company, Rockford, IL). Treatment with hydroxylamine or a 20-h dialysis of the untreated enzyme, resulted in complete inactivation of the enzyme. The activities of the dialyzed treated and untreated enzyme fractions could be restored to 56% and 63%, respectively by addion of PLP. Taken together, these results indicate that PLP is required for enzyme activity.

Table 2 shows that the enzyme has a relatively broad specificity towards sulfur-containing amino acids as measured by keto-acid formation. Degradation of lanthionine, cystathionine, homoserine, cystine, djenkolic acid, cysteine, homocysteine and methionine (in that order of susceptibility) was observed. With the thiol determination method similar results were obtained with the substrates lanthionine, cystathionine, cystine, djenkolic acid and methionine. In all cases, a keto component, ammonia, and a thiol group could be detected. Both cysteine and homocysteine could be identified as degradation products of cystathionine by amino acid analysis. Therefore, it seems that the enzyme is capable of catalyzing both the α,β-elimination and α,γ-elimination reaction. No KMBA was detected with the purified enzyme when methionine was used as a substrate and no thiol formation was detected with KMBA as a substrate. These results suggest a simultaneous deamination and C-S-lyase action (α,γ-elimination) catalyzed by the purified enzyme rather than a coupled reaction mechanism involving two enzymes (and the formation of the intermediate KMBA), which apparently are also

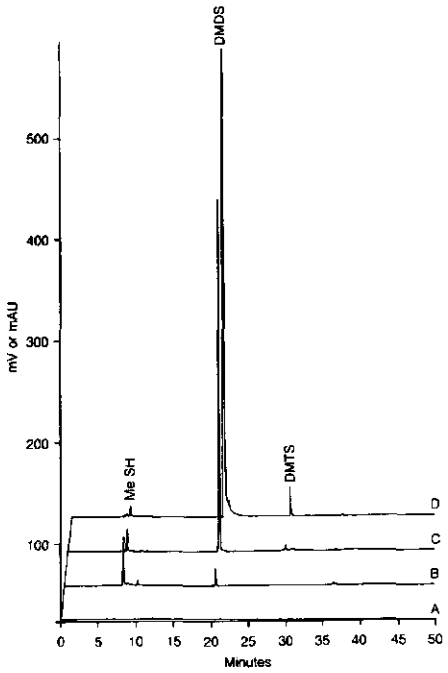

Figure 2. Identification of the volatile (degradation) products of methionine conversion as identified by Thermal-Desorption-Cold-trap-GC after incubation for 120 min (C) or 360 min (D) under the conditions as described in Material and Methods or after an incubation for 360 min without a enzyme addition (B) or without a substrate addition (A). MeSH, methanethiol; DMDS, dimethyldisulfide; DMTS, dimethyltrisulfide.

present in the crude cell-free extract [11]. Deaminase activities involving arginine, glutamine or asparagine, but no other amino acids, were detected in the crude cell-free extract as well [1], but none of these activities were found with the purified enzyme. The deaminase activities found in the cell-free extract are probably due to the action of a specific arginine deiminase [30] and glutaminase-asparaginase [31] respectively, which are enzymes found in many bacteria.

The volatile (degradation) products of methionine conversion were identified by GC as methanethiol, dimethyldisulfide and dimethyltrisulfide (Figure 2). Mainly dimethyldisulfide (but also dimethyltrisulfide) was detected, probably because under aerobic conditions these components are formed from methanethiol. Although the C-S bond in lanthionine is easily split by the purified enzyme, no cleavage of the C-S bonds in nisin A, a peptide containing 5 lanthionine rings, was observed [25]. Apparently a free amino-group is required for α,β-elimination.

The effects of some inhibitors upon the activity of the cystathionine lyase activity are summarized in Table 3. The enzyme was strongly inhibited by carbonyl reagents such as hydroxylamine, DL-penicillamine, phenylhydrazine, semicarbazide and 3-methyl-2-benzothiazolinone hydrazone, which are known inhibitors of PLP-dependent enzymes. Other inhibitors of PLP-dependent enzymes, like DL-cyclo-serine, carboxymethoxylamine (irreversible inhibitor, substrate analog), DL-propargylglycine (irreversible inhibitor) and β-cyano-L-alanine (competitive inhibitor) also strongly

inhibited the enzyme. The sulfhydryl-reactive agents iodoacetic acid, iodoacetamide and *N*-ethylmaleimide caused no marked inhibition of the enzyme. The slight apparent activation is due to the reaction of the sulfhydryl agents with 3-methyl-2-benzothiazolinone used in the keto acid determination. There was also no effect of the chelating agent EDTA. The activity of the enzyme was only slightly reduced by 4% NaCl.

Table 3. Effect of inhibitors on the cystathionine lyase activity.

Inhibitor	Inhibitor Concentration	Relative activity (%)	
		Thiol determination	Keto acid determination
Hydroxylamine	1 mM	1	4
	10 mM	2	0
DL-Penicillamine	1 mM	ND[a]	97
	10 mM	ND	57
Phenylhydrazine	1 mM	19	ND
Semicarbazide	1 mM	91	87
	10 mM	7	48
3-Methyl-2-benzothiazo-	1 mM	ND	91
linone hydrazone	10 mM	ND	53
N-Ethylmaleimide	1 mM	ND	162
	10 mM	ND	151
Iodoacetamide	1 mM	ND	107
	10 mM	ND	136
Iodoacetic acid	1 mM	ND	105
	10 mM	ND	113
Glycine	1 mM	ND	102
	10 mM	ND	94
DL-Propargylglycine	1 mM	1	1
	10 mM	0	0
β-Cyano-L-alanine	1 mM	37	67
	10 mM	6	13
Carboxymethoxylamine	1 mM	67	9
	10 mM	6	0
DL-Cycloserine	1 mM	89	93
	10 mM	51	61
EDTA	1 mM	106	102
	10 mM	94	93
Tris	1 mM	101	105
	10 mM	101	96
NaCl	0.4% (w/v)	100	ND
	4% (w/v)	69	ND

[a] ND, not determined.

4 Discussion

The results in this paper indicate that the enzyme purified from *L. lactis* subsp. *cremoris* B78 is cystathionine β-lyase (EC 4.4.1.8), a PLP-dependent enzyme which is involved in the biosynthesis of methionine. It is responsible for the degradation of cystathionine into homocysteine, pyruvate and ammonia. Subsequently, homocysteine is methylated to form methionine [21].

Although this micro-organism, like most *L. lactis* strains, is auxotrophic for the amino acid methionine, there are indications that a biosynthetic route for methionine exists in *L. lactis*, but it is probably interrupted [6]. The existence of gene defects in *L. lactis* related to the biosynthesis of amino acids has been established by comparing the nucleotide sequence of some of these genes of a non-dairy strain that is prototrophic for the amino acids leucine, isoleucine, valine, and histidine with the homologous sequences from auxotrophic dairy strains. The auxotrophy seems to be the result of accumulated mutations and deletions within these genes [6, 9, 20]. There are also indications that some *L. lactis* subsp. *lactis* strains are able to grow in a methionine-deficient medium at a very low growth rate, suggesting that these strains possess all the genes needed for the synthesis of this amino acid [6]. With a single-step mutagenesis Deguchi and Morishita [8] were able to restore the ability to synthesize certain amino acids in *L. lactis* auxotrophic for these amino acids.

To our knowledge, a cystathionine β-lyase from lactococci has not been described before. The enzyme has been purified from the Gram-negative bacteria *Escherichia coli* [10, 38], *Salmonella typhimurium* [23, 35] and *Bordetella avium* [19], and has been partly purified from *Paracoccus denitrificans* [5]. Analogous to the cystathionine β-lyases occurring in these bacteria, the lactococcal enzyme is a tetrameric protein with identical subunits of approximately 40 kDa in size. The amino-terminal sequence determination of the protein extracted from the 40 kDa-band (SDS-PAGE [12.5 % polyacrylamide]) unequivocally showed a single amino-terminal sequence, indicating that the 40 kDa-band is free of other proteins (data not shown). No sequence similarity could be found between this amino-terminal sequence of the lactococcal enzyme and the amino-terminal sequences of cystathionine β-lyase from *E. coli* [3], *B. avium* [19] and *S. typhimurium* [35]. However, it should be mentioned that the amino-terminal region of the enzyme in these Gram-negative bacteria is not very conserved. At the moment, the amino-terminal sequence is being used to pick up the gene encoding this lactococcal cystathionine β-lyase.

The enzyme has an alkaline pH-optimum, and like many other enzymes of biosynthetic pathways, it is not endowed with an absolute specificity. The relative activity exhibited by the lactococcal enzyme towards different substrates was found to decrease in the order lanthionine > cystathionine > homoserine > cystine > djenkolic acid> (homo-)cysteine > methionine. This order of preference for the different substrates and the identification of cystathionine degradation products show that the enzyme prefers to catalyze the α,β-elimination reaction, but it is capable of catalyzing an α,γ-

elimination reaction as well. The preference for lanthionine, cystathionine, cystine and djenkolic acid is in accordance with the results found for other known bacterial cystathionine β-lyases [5, 10, 19, 23, 35, 42]. It is known that cystathionine γ-lyase isolated from *Streptomyces phaeochromogenes* also catalyzes the α,β-elimination reaction at about one-seventh the rate of the α,γ-elimination reaction [34]. The *Paracoccus* enzyme differs from the lactococcal enzyme by not degrading cysteine, homoserine and methionine [5].

The lactococcal enzyme is very sensitive to propargylglycine and β-cyanoalanine, which are also strong inhibitors of mammalian cystathionine γ-lyase [41]. In contrast to the lactococcal enzyme, the cystathionine β-lyases of *E. coli* and *B. avium* are not inhibited by propargylglycine. Carboxymethoxylamine, a strong inhibitor of PLP-dependent S-alkyl-L-cysteine lyase of *Acacia farnesiana* [32], also strongly inhibits the lactococcal enzyme. Like most of the PLP-dependent enzymes, the enzyme is very sensitive to carbonyl reagents. The chelating agent EDTA and sulfydryl reagents have no effect upon the activity of the lactococcal enzyme, showing that metal ions and thiol groups are not essential for its activity. In this respect the *L. lactis* enzyme differs from the *P. denitrificans* and *B. avium* enzymes, which are both sensitive to sulfydryl reagents. Moreover, under the same experimental conditions the lactococcal enzyme is heat stable within the range 20 to 60°C, whereas the *Bordetella* enzyme is heat stable only up to 40°C [19].

The impact of sulfur-containing compounds on the development of flavour in cheese has been discussed and is widely accepted [29]. The importance of methanethiol and dimethyldisulfide has been emphasized, but several other sulfur compounds have also been identified [7]. Green and Manning [22] have investigated the volatile sulfur compounds in Cheddar cheese and have concluded that methanethiol is important in the development of distinctive Cheddar-like flavours. However, the nature of its flavour-conferring properties has not yet been elucidated. Methionine is accepted as the most important precursor for methanethiol in cheese. It was possible to achieve an improvement of the Cheddar flavour by incorporation of a free or fat-encapsulated methanethiol-generating methionine γ-lyase-methionine system into Cheddar cheeses. However, it was concluded that methanethiol alone did not cause the true Cheddar flavour [29]. Recently, Engels and Visser [13] showed that the cell-free extract of *L. lactis* subsp. *cremoris* B78 is capable of generating a Gouda cheese-like flavour from methionine. Contrary to other authors [27], we associate methanethiol production in cheese with cystathionine β-lyase activity and other enzymes of the lactic starter bacteria in the first place and not with enzyme activities derived from non-starter bacteria or with non-enzymic reactions.

The cystathionine β-lyase is still active at the pH of a normal Gouda cheese and even the presence of a high salt concentration did not strongly inhibit its activity. The activity of the present enzyme in intact, but energetically exhausted [39] cells involving internal (generated) substrate or substrate that enters the cell by passive diffusion is probably limited. Lysis of cells is therefore a prerequisite to the full realization of the enzyme

activity with respect to cheese ripening. In a 5-month-old cheese, some of the cells showed a locally degraded cell wall and a seemingly intact membrane [40]. These cells probably did not burst, because of an osmotically and structurally determined stabilization by the cheese environment [38]. Even in the case of a clear membrane rupture, the cell content remained largely within the cell-envelope structure [40], because of the semi-solid gel structure in which the cell is embedded. Therefore, it is not expected that the activity of the present enzyme decreases after lysis of cells in cheese as a result of dilution of the cofactor. In view of this, and of its relatively high stability, and considering the fact that sulfur-containing flavour components have very low threshold values for perception, the enzyme could play an important role in the cheese-ripening process, despite the fact that the conditions in cheese are far from optimal for its activity.

In conclusion, it has been shown that cystathionine β-lyase, of which the function within the growing cell is not primarily related to amino acid catabolism, can be significantly involved in amino acid conversion. In cheese this relatively low activity might still be important if viewed in the light of the often extremely low concentrations at which volatile flavour products of these actions, or subsequent reactions, can contribute to flavour.

5 Acknowledgements

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Chapter 6

Conversion of methionine by enzymes from Lactococcus lactis subsp. *cremoris* B78: formation of the transamination product 4-methylthio-2-ketobutyric acid and purification and characterization of two aminotransferases involved.

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Conversion of methionine by enzymes from *Lactococcus lactis* subsp. *cremoris* B78: formation of the transamination product 4-methylthio-2-ketobutyric acid and purification and characterization of two aminotransferases involved.

Summary

The transamination of methionine by enzymes from the mesophilic starter organism *Lactococcus lactis* subsp. *cremoris* B78 was studied. Two branched-chain aminotransferases, displaying activity towards methionine, were purified and characterized. The enzymes, AT-A and AT-B, both have a molecular mass of approximately 75 kDa and consisted of two identical subunits, each with a molecular mass of approximately 40 kDa. After further purification the molecular mass of AT-A was established to be 41.98 kDa, using electrospray-ionisation mass spectrometry. Like most pyridoxal 5'-phosphate-dependent enzymes, the lactococcal aminotransferases were strongly inhibited by the carbonyl reagents hydroxylamine, cycloserine and carboxymethoxylamine. In addition to branched-chain amino acids and methionine, the enzymes used phenylalanine and, in the case of AT-B, tryptophan and tyrosine as amino-group donor. AT-A and AT-B also had a broad substrate specificity for the amino-group acceptor, α-ketoglutaric acid being the most preferred substrate. The enzymes catalyze the conversion of methionine to 4-methylthio-2-ketobutyric acid, which is subsequently converted to methanethiol and dimethyldisulfide. The formation of these and other volatile sulfur compounds is considered to be very important for the formation of cheese flavour. Both AT-A and AT-B have a rather high optimum temperature, 45-50 °C, and a pH optimum of 8. However, under cheese-ripening conditions (10-15 °C and pH 5.2-5.4) sufficient activity remained for significant conversion of methionine.

1 Introduction

Flavour formation in cheese is a complex process which in many respects is still poorly understood. In ripening cheese the breakdown of proteins is essential for formation of desirable flavour and texture. The process of proteolysis has been investigated extensively [16, 17, 36, 46] and the products eventually formed are peptides and free amino acids [13, 34, 46].

Fractionation studies carried out with Cheddar [3] and Gouda cheese [13] showed that low-molecular-mass components, to a large extent concentrated in the water-soluble fraction (WSF) of cheeses, are of considerable importance for cheese flavour. Apart from amino acids and small peptides, which are considered to be of less importance for

true cheese flavour [13], various volatile components are found in this fraction e.g. amines, aldehydes, alcohols, acids, and sulfur compounds [5, 15, 19, 26, 46]. In part, these components are produced by degradation of amino acids [1, 14, 21, 34]. However, breakdown products of fat and lactose are found in the WSF as well (e.g. fatty acids, ketones) [4, 9, 15].

The degradation of amino acids by non-starter organisms from surface-ripened cheeses, e.g. *Brevibacterium linens* and *Pseudomonas*, and from blue cheese, e.g. *Penicillium roqueforti*, has been reported [21, 26, 30]. Recent research at our laboratory has shown that during the ripening of hard-type cheeses, such as Gouda, enzymes of mesophilic starter lactococci are probably also involved in the conversion of amino acids to aroma components [14]. We detected degradation products of both methionine and leucine after incubation with cell-free extracts of *Lactococcus lactis* subsp. *cremoris* B78. Moreover, an enzyme from this organism, i.e. cystathionine ß-lyase, capable of converting methionine into volatile sulfur compounds, methanethiol, dimethyldisulfide and dimethyltrisulfide, was purified and characterized [1]. Recently, a similar enzyme, cystathionine γ-lyase, was purified from *Lactococcus lactis* subsp. *cremoris* SK11 by Bruinenberg et al. [8]. The products of methionine breakdown are considered to be very relevant for cheese flavour. The presence of volatile sulfur compounds in various cheese types e.g. Parmesan [4], Cheddar [45], Gouda [15], and blue cheeses has been reported [19]. Their formation is usually attributed to the action of enzymes from non-starter organisms in cheese. Tanaka et al. [43] and Soda et al. [42] purified and characterized methionine γ-lyase from *Pseudomonas ovalis*, a pyridoxal 5'-phosphate (PLP) dependent enzyme, which is regarded as a key enzyme in bacterial methionine metabolism. The same enzyme was reported by Collin and Law [10] from *Pseudomonas putida* and by Lindsay and Rippe [30] from *Brevibacterium linens*, respectively. The enzyme catalyzes the simultaneous deamination and dethiomethylation of methionine.

The possible role of aminotransferases in flavour formation in cheeses has received little attention. These PLP-dependent enzymes catalyse the transfer of the amino group from an α-amino acid to an α-keto acid [22, 27, 40]. The transamination of aromatic amino acids by *Brevibacterium linens* was investigated by Lee and Desmazeaud [27]. Recently Yvon et al. [48] reported the purification and characterization of an aminotransferase from *Lactococcus lactis* subsp. *cremoris* NCDO 763 converting aromatic amino acids.

In the present paper the purification and characterization of two aminotransferases from *Lactococcus lactis* subsp. *cremoris* B78, capable of converting methionine, are described. An enzymic pathway for the formation of methanethiol via 4-methylthio-2-ketobutyric acid (α-keto-γ-methylthiobutyric acid, KMBA) is proposed.

2 Materials and methods

2.1 Materials

Amino acids, α-keto acids and the inhibitors hydroxylamine, DL-cycloserine, iodoacetamide, and DL-propargylglycine were purchased from Sigma Chemicals (St. Louis, Mo.). α-Keto glutaric acid was obtained from Janssen Chimica (Geel, Belgium), carboxymethoxylamine and KMBA from Aldrich Chemie GmbH (Steinheim, Germany), and *N*-ethylmaleimide from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

2.2 Organism and growth

Lactococcus lactis subsp. *cremoris* B78 was isolated from DL-type starter, code Bos, and was maintained and grown in milk (overnight at 20 °C). Late-logarithmic phase cells were harvested and washed as described previously [14]. The washed cells were resuspended to an OD_{650nm} of approximately 80 in 20 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 20 μM PLP (20 mM KPi-EDTA-PLP). The suspension was stored overnight at -30 °C.

2.3 Preparation of cell-free extract (CFE)

The -30 °C cell suspension was defrosted and subsequently the cells were disrupted ultrasonically at 0 °C for 15 cycles of 15 s (Heat Systems Sonicator XL 2020, NY, USA). The treated suspension was centrifuged (30 min, 30000 g, 4 °C) to remove intact bacteria and cell debris, and the supernatant (CFE) was collected. CFE was stored at -30 °C until further use.

2.4 Determination of aminotransferase (AT) activities

AT activity, towards methionine, in CFE and in fractions thereof obtained during purification was determined routinely by incubating 100 μl samples in 20 mM or 50 mM (purified enzyme) KPi-EDTA-PLP, pH 7.5, with methionine (final concentration 10 mM) and co-substrate α-ketoglutaric acid (final concentration 5 mM). The final volume of the incubation mixtures was 200 μl. The incubations were performed at 30 °C for 2 hours in the dark. In a preliminary experiment it was established that the AT reaction rate was constant up to 3 hours of reaction. The reaction was stopped by lowering the pH of the mixture to 2.5 via addition of 0.2 M HCl. This procedure will be further referred to as the standard assay.

The formation of KMBA during incubation was quantified by use of high-performance liquid chromatography (HPLC). The HPLC system consisted of an ISS-100 Perkin Elmer automatic sample injector, two Waters M6000 A pumps, an AGC Waters

type 680 gradient controller, and a Kratos 783 UV detector operating at 220 nm. Samples were chromatographed at 30 °C on a Bio-Rad HiPore RP-318 reversed-phase column (4.6 mm × 250 mm) preceded by a Bio-Rad C_{18} cartridge guard column. The elution buffers were 5 % acetonitrile, 0.1 % trifluoroacetic acid (TFA) in water (solvent A) and 90 % acetonitrile, 0.08 % TFA in water (solvent B). The components in the reaction mixture were separated isocratically at 0 % solvent B for 5 min followed by a linear gradient from 0 to 70 % solvent B over 2 min and isocratic elution at 70 % solvent B for 5 min. The flow rate was 0.8 ml/min. KMBA eluted at 9.4 min. KMBA concentrations were determined using a standard curve with KMBA (0.02 - 5 mM) in 20 mM KPi-EDTA-PLP. Perkin Elmer Nelson turbochrom 4.0 software (Cupertino, CA.) was used for processing raw HPLC data.

In preliminary studies a fast thin-layer chromatography (TLC) method for identification of KMBA as described by Alting et al. [1] was used. Solutions of methionine and KMBA were used as standards. R_f values of methionine and KMBA were 0.57 and 0.71, respectively.

The conversion of KMBA to methanethiol, by CFE and fractions thereof, was monitored by determining the methanethiol oxidation product dimethyldisulfide (DMDS), using dynamic headspace gas chromatography with flame-photometric detection of sulfur compounds [1]. Incubations with KMBA (final concentration 4 mM) were in 20 mM KPi-EDTA-PLP, pH 7.5, at 30 °C.

2.5 Purification of aminotransferases

All purification steps were carried out at 4 °C. Fractionation was performed by stepwise addition of solid ammonium sulfate to 80 ml of CFE. Four ammonium sulfate fractions (0-20 % (wt/vol) pellet, 20-37 % (wt/vol) pellet, 37-55 % (wt/vol) pellet and 55 % supernatant) were obtained by adding solid ammonium sulfate. Precipitated protein material was collected by centrifugation at 15000 g for 5 min and dissolved in 20 mM KPi-EDTA-PLP, pH 7.5 (in the original volume). The solution displaying the highest AT activity towards methionine was extensively washed with the buffer and concentrated in a stirred-type Amicon ultrafiltration cell (Amicon, Danvers, MA, USA), operated under a nitrogen pressure of 300 kPa with a 10 kDa molecular mass cut-off Omega membrane (Filtron, Northborough, MA, USA). The washed and concentrated protein sample (15 ml, 6.7 mg protein/ml) was applied to a Q-Sepharose (Pharmacia Biotech, Uppsala, Sweden) anion-exchange column (50 mm × 140 mm) equilibrated with 20 mM KPi-EDTA-PLP, pH 7.5. Proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in 20 mM KPi-EDTA-PLP buffer over a period of 100 min followed by isocratic elution at 0.5 M NaCl for 30 min. A flow rate of 5 ml/min was used and 10 ml fractions were collected. Absorbance was measured at 280 nm with a UV-MII detector (Pharmacia-LKB, Uppsala, Sweden). The active fractions were pooled and concentrated by ultrafiltration as described above. The concentrated enzyme solution (5 ml, 5 mg protein/ml) was loaded onto a MonoQ HR 5/5 anion-exchange column

(Parmacia Biotech) equilibrated with the 20 mM KPi-EDTA-PLP buffer. Proteins were eluted with a linear gradient of 0 to 0.45 M NaCl in 20 mM KPi-EDTA-PLP buffer at 0.5 ml/min over a period of 90 min. Fractions with a volume of 1 ml were collected. Absorbance was measured at 280 nm. AT activity towards methionine was detected in fractions of two different regions of the MonoQ chromatogram. These will be referred to as AT-A and AT-B containing fractions, respectively. Each fraction was analysed for purity by SDS PAGE. The purest active fractions from each of the two peaks were pooled, washed with 50 mM KPi-EDTA-PLP, pH 7.5, and concentrated by ultrafiltration as described above. The sample containing AT-A was stored at -20 °C. The concentrated sample containing AT-B was loaded onto a Superose 12 HR 10/30 gel filtration column (Pharmacia Biotech) and eluted with 50 mM KPi-EDTA-PLP, pH 7.5 at a flow rate of 0.3 ml/min. The AT active fractions were collected and stored at - 20 °C.

2.6 Analytical determinations

Purity of the enzymes was monitored by polyacrylamide gel electrophoresis (PAGE). For native PAGE, 8 to 25 % Phastgel gradient gels were used with the Phast System (Pharmacia Biotech, Uppsala, Sweden) and 4 to 15 % gradient Mini Protean II Ready Gels with a Mini Protean II cell (Bio-Rad Laboratories, Hercules, CA.). Sodium dodecyl sulfate (SDS) PAGE was performed on 12.5 % polyacrylamide gels with either the Phast System or the Mini Protean II cell. Proteins from Pharmacia low- and high-molecular-mass calibration kits were used as molecular mass markers.

Isoelectric points were determined using linear pH gradient gels in the range 4 to 6.5 (Pharmacia Phastgel IEF 4-6.5) with the Pharmacia Phast System.

Proteins on both PAGE and IEF gels were detected by using Coomassie Brilliant Blue R-250 or silver staining (Pharmacia). Electrospray-ionisation mass spectrometry (ESI-MS) (Micromass Quattro II, Cheshire, UK) and amino terminal protein sequencing (Hewlett Packard G1005A micro sequencer, Palo Alto, CA) were conducted on protein fractions obtained by reversed-phase (RP) HPLC. The HPLC equipment used was described above, with 10 % acetonitrile and 0.1 % TFA in water (buffer A) and 90 % acetonitrile and 0.08 % TFA in water (buffer B) as solvent system. Proteins were separated by a linear gradient of buffer B in buffer A from 20 to 50 % buffer B over 30 min, from 50 to 70 % buffer B over 5 min and isocratic at 70 % buffer B for 5 min. The flow rate was 0.8 ml/min.

2.7 Protein determination

Protein concentrations were estimated by the method of Bradford [6] with the Pierce test (Pierce, Rockford, Il, USA) using bovine serum albumin as standard (fraction V, Pierce).

2.8 Dependence of activity on temperature and pH

The activities of AT-A and AT-B, in the purified MonoQ fractions, towards methionine and α-ketoglutaric acid were determined at temperatures ranging from 10 to 70 °C in 50 mM KPi-EDTA-PLP (pH 7.5) using the standard assay. Dependence on pH, with methionine and α-ketoglutaric acid as substrates, was assayed in Britton and Robinson universal buffer [7] at 30 °C.

2.9 Substrate specificity

Activity of AT-A and AT-B towards α-ketoglutaric acid and the various amino acids tested was determined by incubation of AT-containing MonoQ fractions (20 µg protein) with the amino acids (final concentration 10 mM) and α-ketoglutaric acid (final concentration 5 mM) at 30 °C for 2 hours in the dark in 50 mM KPi-EDTA-PLP, pH 7.5. The decrease of the concentration of the amino acids, as a result of AT activity, was measured with a 4151 Alpha Plus amino acid analyser (Pharmacia-LKB, Uppsala, Sweden). The standard aminotransferase assay was used to measure activity of AT-A and AT-B (20 µg protein in assay) towards methionine and various α-keto acids. A final concentration of 5 mM was used for each α-keto acid.

2.10 Effects of inhibitors on the activity

The effects of several inhibitors were determined by studying AT-A and AT-B activity towards methionine and α-ketoglutaric acid, using the standard assay, after preincubation of the enzymes for 30 min with the inhibitors (each at a concentration of 1 mM).

3 Results

3.1 Formation of KMBA and DMDS from methionine by enzymes in CFE of Lactococcus lactis subsp. cremoris B78

Methionine was incubated, in the presence of α-ketoglutaric acid and PLP, with various CFE fractions obtained by ammonium sulfate precipitation. Equal volumes of the CFE fractions were used. Both single fractions, as mentioned in Materials and methods, as well as combinations of these fractions, were used in the incubations (Table 1). Production of the volatile sulfur compound DMDS was measured by gas chromatography.

The amount of DMDS formed during incubation of total CFE with methionine and α-ketoglutaric acid was about 3 µmol/l (Table 1), no single CFE fraction being capable of forming similar amounts of DMDS. Only the combined 20-37 % and 37-55 % ammonium sulfate fractions were able to generate the same level of DMDS. TLC re-

Table 1. Formation of DMDS from methionine or KMBA by CFE fractions obtained by precipitation with ammonium sulfate[1].

CFE fraction	DMDS formation (μmol/l)	
	Substrate methionine (10 mM)	Substrate KMBA (4 mM)
0-20 % $(NH_4)_2SO_4$ pellet	0.23	-[2]
20-37 % $(NH_4)_2SO_4$ pellet	0.50	1.26
37-55 % $(NH_4)_2SO_4$ pellet	0.41	0.08
0-20 % + 20-37 % $(NH_4)_2SO_4$ pellet	0.73	1.49
0-20 % + 37-55 % $(NH_4)_2SO_4$ pellet	0.71	0.18
20-37 % + 37-55 % $(NH_4)_2SO_4$ pellet	2.89	0.99
CFE	3.06	0.79

[1] Incubations performed at 30 °C under standard assay conditions with α-ketoglutaric acid (5 mM) as cosubstrate. Pellets dissolved in equal volumes of 20 mM KPi-EDTA-PLP, pH 7.5, as described in Materials and Methods.

[2] Not detected.

vealed formation of KMBA during these incubations and during incubation with the single 37-55 % ammonium sulfate fraction. These data led to the conclusion that *Lactococcus lactis* subsp. *cremoris* B78 degrades methionine by a two-step mechanism in which KMBA is an intermediate. The 37-55 % fraction is required for the formation of KMBA from methionine, whereas the 20-37 % ammonium sulfate fraction facilitates the breakdown of KMBA. In an additional experiment it was shown that substantial formation of DMDS from KMBA (4 mM) only occurred in the presence of the 20-37 % ammonium sulfate fraction (Table 1).

The formation of KMBA, as well as the requirement of α-ketoglutaric acid, suggested involvement of aminotransferases, present in the 37-55 % ammonium sulfate fraction. The removal of PLP from this fraction by ultrafiltration drastically reduced the formation of KMBA.

3.2 Purification and characterization of aminotransferases

The 37-55 % ammonium sulfate fraction was subjected to further fractionation by Q-Sepharose chromatography and anion-exchange chromatography on a MonoQ HR 5/5 column (Figure 1). Fractions 16 - 18 and 26 - 28 contained two aminotransferase activities, AT-A and AT-B, respectively. The results of the purifications of the AT-A- and AT-B-containing fractions, based on the assay of methionine aminotransferase ac-

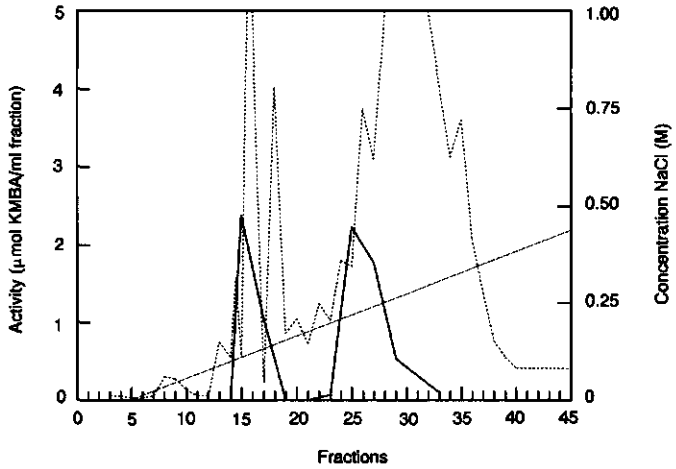

Figure 1. Resolution on MonoQ HR 5/5 of two aminotransferase activities (——) towards methionine. Fractions (1 ml) were collected and assayed for activity using α-ketoglutaric acid as cosubstrate under standard assay conditions. Displayed are A_{280}, ·····, and concentration NaCl, -----.

tivity, are shown in Tables 2 and 3. The level of purification of AT-A was 49-fold, with an activity yield of 0.9 %, and the level of purification of AT-B, achieved with an additional gel filtration step, was 229-fold (yield 0.3 %).

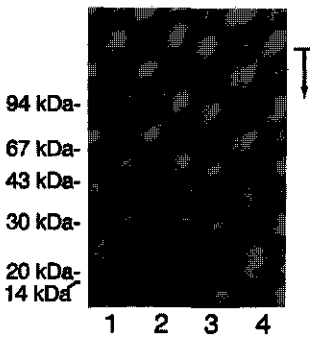

Figure 2. SDS-PAGE in combination with silver staining showing purified AT-A (lane 3) and AT-B (lane 2). Lanes 1 and 4: Pharmacia low-molecular-mass markers (see Materials and methods).

As judged by SDS-polyacrylamide-gel electrophoresis both AT-A- and AT-B-containing fractions were reasonably homogeneous, each showing an apparent molecular mass near 40 kDa (Figure 2). However, the presence of several components in the purified fractions was detected by RP-HPLC (results not shown), and by isoelectric focusing. After applying silver staining, the isoelectric focusing gel showed four bands for AT-A and three bands for the AT-B containing fraction (not shown). The bands were cut out of the gels, the protein was extracted with 50 mM KPi-EDTA-PLP, pH 7.5, and AT activity towards methionine was determined. The gel administered with the AT-A-containing fraction displayed AT activity in a protein band with a pI value of 4.45.

Table 2. Purification of AT-A from *Lactococcus lactis* subsp. *cremoris* B78.

Purification step	Total protein (mg)	Total activity (U[1])	Specific activity (U/mg)	Purification (fold)
Cell-free extract	703	459	0.6	1.0
Ammonium sulfate fraction 37-55 %	100	104	1	1.5
Q-Sepharose	31	44	1.2	1.9
MonoQ	0.3	3	31	48.6

[1] One unit (U) catalyzes the formation of 1 µg of KMBA per min.

Table 3. Purification of AT-B from *Lactococcus lactis* subsp. *cremoris* B78.

Purification step	Total protein (mg)	Total activity (U[1])	Specific activity (U/mg)	Purification (fold)
Cell-free extract	859	503	0.6	1.0
Ammonium sulfate fraction 37-55 %	95	74	0.8	1.4
Q-Sepharose	32	44	1.4	2.4
MonoQ	1	15	21	35.7
Superose 12	0.01	1.5	136	229

[1] One unit (U) catalyzes the formation of 1 µg of KMBA per min.

With the AT-B-containing fraction, a protein band with a pI value of 4.55 showed AT activity.

For further purification of AT-A, the four subfractions obtained by IEF were subjected to RP-HPLC. In Figure 3 the RP-HPLC chromatograms are shown as well as the chromatogram of the whole AT-A-containing MonoQ fraction. From the fact that trace A represents the only IEF fraction having AT-A activity, it was concluded that this activity should be ascribed to component 2 rather than to component 1 (Figure 3). Both components were isolated and their amino-terminal sequence and molecular mass (ESI-MS) were determined. The N-terminal sequence of component 1 showed a high degree of homology to that of glyceraldehyde-3-phospate dehydrogenase (EC 1.2.1.12). The molecular mass of this protein was determined to be 35.75 kDa. The N-terminal sequence of component 2 is A K L T V K D V E L K G K K V L V R V D, while its molecular mass was found to be 41.98 kDa. These values are in agreement with the molecular mass of the corresponding AT-A-containing MonoQ fraction, estimated by SDS PAGE (with both component 1 and 2 appearing in one band, Figure 2). On native PAGE a single 75 kDa band, displaying AT activity towards methionine, was observed (results not shown)

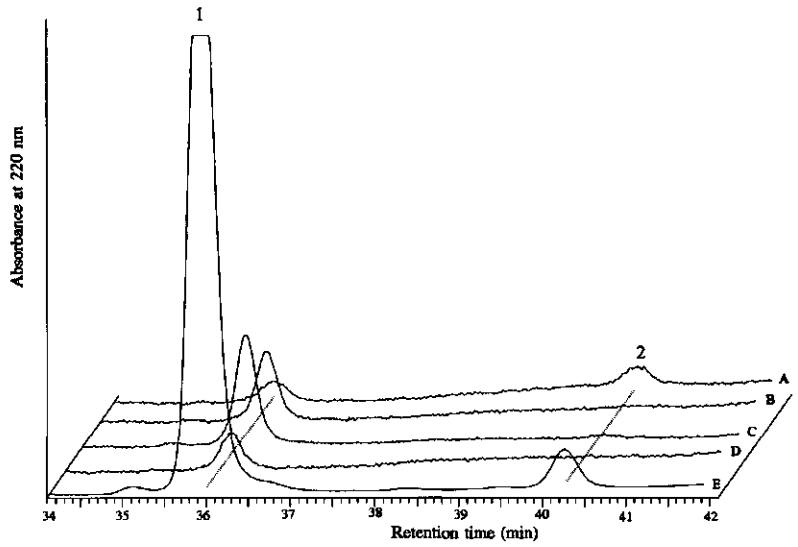

Figure 3. Reversed-phase high performance liquid chromatograms of the total MonoQ AT-A-containing fraction (trace E) and of subfractions purified from the IEF gel (traces A - D). 1 = glyceraldehyde-3-phosphate dehydrogenase, 2 = AT-A.

3.3 Temperature and pH dependence

For the transamination of methionine, the optimum pH was 8, for both AT-A and AT-B (Figure 4). AT-A and AT-B exhibited a rather broad range of maximum activity between the pH values 7 and 8.5. At the pH of cheese (5.2) about 20 % of the enzyme activity found at the optimum pH was observed.

The influence of the temperature on the reaction rate is shown in Figure 5. The optimum temperature for methionine transamination by AT-A and AT-B was 45-50 °C. At temperatures above 50 °C the activity decreased rapidly.

3.4 Substrate specificity

Table 4 shows the relative activities of AT-A and AT-B towards various amino acids and α-keto acids. The enzymes showed a relatively broad specificity towards both α-keto acids (particularly α-ketoglutaric acid, α-ketobutyric acid, and α-ketopentanoic acid) and amino acids. Valine, isoleucine, leucine, and methionine were the preferred amino acids, suggesting mostly branched-chain aminotransferase activity. AT-B also showed substantial activity towards aromatic amino acids.

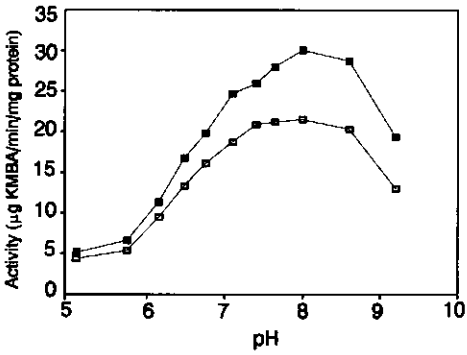

Figure 4. pH dependence of aminotransferase activity towards methionine and α-ketoglutaric acid. Levels of AT-A (■) and AT-B (□) were determined using the standard assay with Britton and Robinson universal buffer [7] (see Materials and methods).

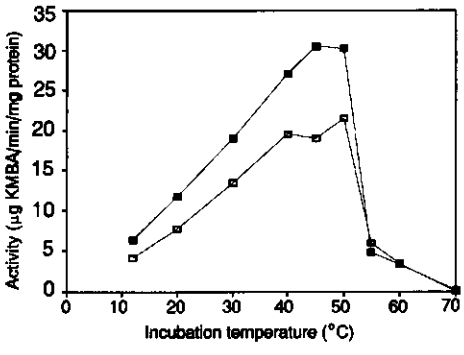

Figure 5. Temperature dependence of AT-A (■) and AT-B (□) activity towards methionine and α-ketoglutaric acid. Activity was determined by the standard assay (see Materials and methods).

Table 4. Substrate specificity of the aminotransferases AT-A and AT-B.

Substrate	Relative activity (%)	
	AT-A	AT-B
Amino donor[1]		
L-valine	323	376
L-isoleucine	307	385
L-leucine	282	327
L-methionine	100	100
L-phenylalanine	12	38
L-tyrosine	-[3]	24
L-tryptophan	-	24
L-aspartic acid	-	1
L-histidine	-	<1
Amino acceptor[2]		
α-ketoglutaric acid	100	100
α-ketobutyric acid	78	75
α-ketopentanoic acid	64	60
α-ketoadipinic acid	45	43
phenylpyruvic acid	38	44
pyruvic acid	12	12
oxalacetic acid	4	5

[1] 5 mM α-ketoglutaric acid was used as amino-group acceptor. The concentrations of the amino acids were 5 mM, except for tyrosine, which was assayed at 2 mM. Activity on methionine was adjusted to 100 %.

[2] 10 mM methionine was used as amino-group donor. All α-keto acids were assayed at 5 mM. Activity on α-ketoglutaric acid was adjusted to 100 %.

[3] Not detected.

3.5 Inhibitors

AT-A and AT-B were strongly inhibited by the carbonyl binding agents hydroxylamine, carboxymethoxylamine and cycloserine (Table 5). The thiol reagents iodoacetamide and *N*-ethylmaleimide caused only a minor inhibition of AT-A and AT-B activities when utilized at a concentration of 1 mM. At a concentration of 3 mM, however, *N*-ethylmaleimide caused considerable inhibition. Propargylglycine and EDTA had no inhibitory effect, whereas NaCl showed inhibition of aminotransferase activity only when present at a high concentration (0.85 M).

Table 5. Effects of inhibitors on activity of AT-A and AT-B.

Inhibitor	Inhibitor concentration	Remaining activity (%)[1]	
		AT-A	AT-B
hydroxylamine	1 mM	2	5
cycloserine	1 mM	13	16
carboxylmethoxylamine	1 mM	2	3
iodoacetamide	1 mM	85	80
N-ethylmaleimide	1 mM	99	87
propargylglycine	1 mM	99	92
EDTA	4 mM	100	99
NaCl	0.17 M	98	101
	0.85 M	89	86

[1] The level of activity observed in the absence of inhibitor was defined as 100 %.

4 Discussion

The data presented in this study demonstrate that methionine can be a substrate for aminotransferases (ATs) from *Lactococcus lactis* subsp. *cremoris* B78. Two fractions with AT activity, AT-A and AT-B, were resolved by anion-exchange chromatography. Both ATs were also able to transaminate various other amino acid substrates, such as leucine, isoleucine, valine, and phenylalanine, and displayed dependence on PLP and on an α-keto acid cosubstrate. The higher activity towards the branched-chain amino acids leucine, isoleucine, and valine suggests that branched-chain ATs have been purified [24, 29]. A broad substrate specificity, however, is a common feature of ATs and conversion of methionine by branched-chain aminotransferases of other origin has been reported [24, 25, 39].

ATs are pyridoxal-5'-phosphate (PLP) dependent enzymes that catalyze the reversible transfer of the amino group from an α-amino acid to an amino acceptor, primarily an α-keto acid [2]. The reaction catalyzed by ATs occurs as the result of a two-step reaction. The first step involves transfer of the amino group of the amino acid to PLP to yield an α-keto acid product and an enzyme-bound pyridoxamine-5'-phosphate. During the second step, the amino group from pyridoxamine-5'-phosphate is transferred to an α-keto acid to produce an amino acid and regenerate PLP [37]. Removal of PLP from AT-A- and AT-B-containing fractions by ultrafiltration largely depleted the enzyme activities. This demonstrates the role of PLP as a cofactor.

Apart from the difference in elution behaviour during MonoQ anion-exchange chromatography, the AT-A and AT-B containing fractions differed in amino acid substrate specificity. AT-B was able to transaminate not only branched-chain amino acids, methionine and phenylalanine, but also tyrosine and tryptophan. Regarding the specificity towards the α-keto acids, there was hardly any difference between AT-A and AT-B, α-ketoglutaric acid being the most preferred substrate for both enzymes. In contrast with a branched-chain aminotransferase purified from *E. coli* [24], the lactoccal enzymes also utilized oxalacetate and pyruvic acid as amino acceptors. Both AT-A- and AT-B-containing fractions showed a 40 kDa band on SDS PAGE, whereas with native gradient PAGE a 75 kDa band was seen. This suggests that the ATs are dimeric proteins with identical subunits. In *E. coli* [24] and in mammals [25] branched-chain ATs with similar structural properties were found. With ESI-MS the subunit molecular mass of AT-A was estimated to be 41.98 kDa. A protein having N-terminal sequence identity with glyceraldehyde-3-phosphate dehydrogenase, molecular mass 35.75 kDa, was obviously co-purified with AT-A. After purification by IEF this protein appeared to possess no AT activity. The N-terminal amino acid sequence of AT-A showed no homology with known branched-chain ATs. However, we did find fairly high homology with 3-phosphoglycerate kinase and therefore the presence of such an enzyme could not be completely ruled out. Currently, the amino-terminal sequence is being used to pick up the gene encoding the enzyme. An overproducing strain will be constructed and will be tested for its aminotransferase activity.

Both AT-A and AT-B had a rather high optimum temperature and an alkaline pH optimum. These findings are in accordance with results found for other ATs [24, 25, 38]. The pI values of AT-A and AT-B were comparable with both the pI values of branched-chain ATs of *Methanococcus aeolicus* [47] and *Bacillus brevis* [24] as well as with those of aromatic ATs of *Thermococcus litoralis* [2].

Like most PLP-dependent enzymes, the ATs were strongly inhibited by the carbonyl reagents hydroxylamine, cycloserine, and carboxymethoxylamine, presumably by their reaction with the aldehydic moiety of PLP [20]. Branched-chain ATs from *E. coli* [24] and mammals [25] showed equal sensitivity towards these reagents. Propargylglycine, a strong inhibitor of cystathionine lyases [1], had no effect on the activity of the lactococcal ATs. Only a modest inhibition by thiol reagents (iodoacetamide and *N*-

ethylmaleimide), EDTA, and NaCl was observed, which is in agreement with findings for other ATs [38].

Methionine plays a central role in the catabolism of sulfur-containing amino acids. In vertebrates, the transsulfuration pathway is known [11, 41]. This route comprises the conversion of methionine to cystathionine through *S*-adenosylmethionine and homocysteine. For microorganisms a similar pathway has been characterized and one of the enzymes involved is cystathionine ß-lyase. Previously, we reported the purification of this enzyme from *Lactococcus lactis* subsp. *cremoris* B78 and its possible relevance for cheese flavour formation. At that time we also observed the formation of the transamination product KMBA from methionine with cell extracts of *Lactococcus lactis* subsp. *cremoris* B78 [1]. From the results of the present incubation experiments performed with single as well as combined ammonium sulfate precipitation fractions we conclude that at least two enzymic steps are involved in transaminase-catalyzed degradation of methionine (Figure 6). The first step, during which KMBA is produced, is the actual transamination step. This conversion of methionine is probably a secondary function of the presently described branched-chain ATs. The intermediate KMBA is eventually converted to methanethiol, which under aerobic conditions is probably converted rapidly to DMDS and/or DMTS (Figure 6). Transaminations are physiologically very important because they play a crucial role in the biosynthesis as well as the catabolism of amino acids [18, 32, 44]. In nutritionally fastidious bacteria, like lactic acid bacteria, the interconversion of amino acids by ATs may be necessary when the organisms are grown on media not supplying the optimal proportion of amino acids.

Transamination during cheese ripening has been reported. However, mostly organisms from surface-ripened cheese, e.g. *B. linens* [21, 28] were implicated. A similar route of degradation of methionine, leading to the formation of ethylene, methanethiol, and carbon dioxide, has been proposed in *E. coli* by Ince and Knowles [22] as well as Shipston and Bunch [40]. Morgan [33] described the conversion of branched-chain amino acids by transamination, and the subsequent conversion of the keto acids formed to aldehydes, due to the metabolic activity of *Lactococcus lactis* var. *maltigenes*. In the case of methionine, the aldehyde formed, by decarboxylation of KMBA in the presence of thiamin pyrophosphate, would be methional. Methional has been detected in Cheddar [45] and Emmental cheese [35]. With the aid of gas chromatography-mass spectrometry we could also detect the formation of methional during incubation of CFE from *Lactococcus lactis* subsp. *cremoris* B78 with methionine (results not shown). Recently, a paper was published by Yvon et al. [48] describing the purification and characterization of an aromatic amino acid converting aminotransferase from *Lactococcus lactis* subsp. *cremoris* NCDO 763. The enzyme is able to convert aromatic amino acids, but also leucine and methionine. However, the specificity towards various amino acids, as well as the specificity towards α-keto acids, is clearly different from those of our enzymes. For instance, the branched-chain amino acids isoleucine and valine did not appear to be suitable substrates for the aminotransferase.

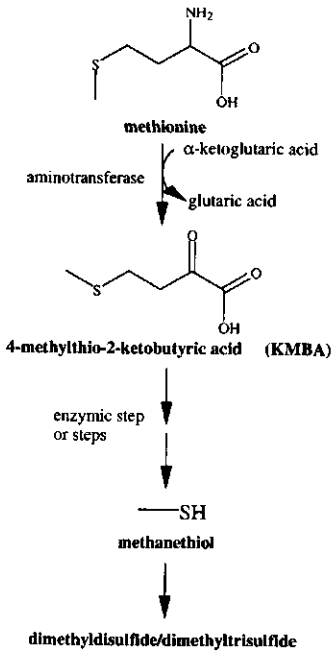

Figure 6. Proposed pathway of the formation of methanethiol from methionine by enzymes from *Lactococcus lactis* subsp. *cremoris* B78.

The formation of volatile sulfur compounds is considered to be very important for formation of cheese flavour [12, 14, 23]. Previously, the breakdown of methionine, and other amino acids, was frequently attributed to non-starter organisms. Furthermore, in hard-type cheeses, like Cheddar, the breakdown of methionine, and the subsequent formation of sulfur volatiles, was considered mainly to be a non-enzymic process favoured by the low redox potential in the cheese interior [31]. The present study demonstrates the importance of enzymes from mesophilic lactococci in the process of methionine conversion. Together with the degradation of other amino acids by the ATs characterized, but also by other enzymes, this may lead to a myriad of breakdown products in the ripening cheese, many of them contributing to cheese flavour. A prerequisite for the above processes to take place is of course the availability of free amino acids. This requires a balanced proteolysis in cheese as well as lysis of starter cells to some extent [46]. The conditions in the cheese interior, e.g. low oxygen, low moisture, high salt, will naturally influence the amino acid conversion processes. However, our results show that in the case of the ATs, and in the case of cystathionine-ß-lyase [1], sufficient activity remains for a significant conversion of methionine.

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Chapter 7

Flavour formation in cheese-like systems: role of methionine-degrading enzymes from lactococci

Flavour formation in cheese-like systems: role of methionine-degrading enzymes from lactococci

1 Introduction

Investigations described in the chapters 2 to 6 of this thesis have demonstrated that non-proteolytic enzymes from mesophilic starter lactococci may play an essential role in the formation of flavour in cheese. In this respect, special attention was paid to the enzymic breakdown of methionine, which appeared to be an important precursor of cheese flavour. The formation of sulphur-containing products, like methanethiol, dimethyldisulphide and dimethyltrisulphide, from this amino acid by enzymes from *Lactococcus lactis* subsp. *cremoris* B78 was demonstrated [4]. The purification and characterization of cystathionine ß-lyase [1] and two aminotransferases [3] involved in methionine degradation was established. Moreover, during incubations in a model system, the formation of volatile sulphur compounds was accompanied with the formation of cheese-like flavours [4].

To validate the role of starter enzymes in combination with amino acids under more practical conditions, we have performed some exploratory experiments during which both the formation of flavour and the formation of volatile sulphur compounds were investigated in cheese-like systems to which methionine and cell-free extract (CFE) of *Lactococcus lactis* subsp. *cremoris* B78 had been added. For this purpose, two model systems were developed: (1) a system consisting of a cheese paste, produced from Gouda cheese and (2) a system in which a cheese curd was used. The use of cheese model systems, e.g. slurry or curd systems, to study flavour development is not new. In 1967, Kristoffersen et al. already reported development of Cheddar flavour in such a system [7]. More recently cheese-based model systems were proposed by Smit et al. [10], Youssef [11] and Roberts et al.[9]. Since the conditions in cheese can be closely simulated, the use of these systems is valuable for assessing the role of organisms or their enzymes in cheese ripening.

2 Materials and methods

2.1 Cheese paste experiments

2.1.1 Production of cheese paste. Young Gouda cheese (age 2 weeks, moisture content 38 %, salt in dry matter 1.7 %) was used as the basic material for the preparation of cheese paste. The cheese was shredded and pasteurized by heating to 80 °C for 5 min. Whey proteins (WPC-70, NIZO, Ede, the Netherlands, 10 mg/g paste) were added in order to increase meltability. The cheese paste produced in this way had a low salt content (1.4 % in dry matter) and a water content similar to that of Gouda cheese (43 %). As a result of the pasteurization step, enzymes from starter bacteria and from other

sources were inactivated, although some residual enzyme activity, e.g. by plasmin from milk, could not be excluded. The cheese paste was stored under aseptic conditions at -2 °C until further use.

2.1.2 Incubation of cheese paste with CFE from Lactococcus lactis *subsp. cremoris B78 and methionine.* Cells from an overnight-grown milk culture of *Lactococcus lactis* subsp. *cremoris* B78 (NIZO collection, number B358) were harvested and lysed ultrasonically as described before [4]. The CFE obtained was ultrafiltered to remove low-molecular-mass components. Ultrafiltration was performed at 4 °C in a stirred-cell type ultrafiltration module (Amicon Corporation, MA, USA), operated under a nitrogen pressure of 300 kPa using Amicon Diaflo membranes YC05 (500 Da molecular mass cut-off, MMCO). The retentate (CFE>500) contained the CFE protein material and its volume was reduced to 25 % of the original volume, after being repeatedly washed and refiltered with distilled water.

The formation of flavour in cheese paste was studied by adding 2.5 ml filter-sterilized (Millex-GS, 0.22 µm, Millipore, Molsheim, France) samples of CFE>500, methionine in distilled water (4 mg/ml) or CFE>500 plus methionine (4 mg/ml) to 60 g of aseptically ground cheese paste. A blank incubation was performed with 2.5 ml distilled water added to 60 g of cheese paste. After addition, each portion of cheese paste was thoroughly mixed and subsequently stored under vacuum in a sealed Allvac bag (Pie, Rotterdam, Netherlands) at 18 °C for 2 weeks.

2.2 Cheese curd experiments

2.2.1 Production of cheese curd. Cheese curd was produced from 200-litre portions of pasteurized (10 s, 72 °C) milk in the manner which is usual for Gouda cheese. After cutting the coagulum, whey was drained off and the curd was washed (temperature 32 °C) and subsequently stirred for 30 min. Finally, the curd particles were collected from the whey and divided into 5 kg portions. To these portions CFE and/or methionine were added.

2.2.2 Preparation of CFE and application in cheese curd. Lactococcus lactis subsp. *cremoris* B78 was grown in pasteurized (30 min, 100 °C) milk, reconstituted from spray-dried, non-fat milk powder (10 % w/v), in a 20 l fermentor (Vasal, France). The pH of the medium was kept at 6.5 by titration with 10 % (w/v) $NaHCO_3$ and 7.5 % NH_4OH. Anaerobic conditions were achieved by flushing with N_2.

The cells were harvested at the late exponential growth phase (OD_{650} = 20) by centrifugation (40 min, 4 °C, 7,000 g, Hermle ZV630, BHG, Gosheim, FRG) after clarification of the milk with 1 % (w/v) sodium citrate, and washed twice with 0.1 M sodium phosphate buffer (pH 6.8). Washed cells were resuspended to an OD_{650} of approximately 360 in distilled water. CFE was prepared by passing the cell suspension 4 times through a Rannie-APV (Rannie, Copenhagen, Denmark) homogenizer (model

mini-lab, type 8.30H) at 800 bar. Cell debris and intact bacteria were removed by centrifugation at 15,000 *g* and 4 °C for 30 min. Judged by the release of the intracellular enzyme lactate dehydrogenase (LDH) the treatment with the Rannie homogenizer appeared to be effective for obtaining cell disruption.

To the 5 kg curd portions additions (125 ml) of CFE, CFE + methionine (900 mg) and distilled water + methionine (900 mg) were made aseptically (in duplicate to facilitate organoleptic assessment after both 6 weeks and 13 weeks). After mixing, the curds were pressed, brined (64 hours) and ripened (13 °C). One curd portion with 125 ml distilled water to secure an equivalent moisture content was considered as a control.

2.3 Analysis

2.3.1 Control analysis. Compositional analyses for fat, salt, pH and moisture on the ripening curds were performed according to standard procedures.

After 14 days of incubation, the cheese pastes were analysed for contaminating microflora by homogenizing 10 g of paste with 90 ml of a 2 % solution of sodium citrate in sterile stomacher bags (Seward Medical, London, UK). After dilution the formation of colonies on TGV agar [6] was examined.

2.3.2 Sensory analysis. After 2 weeks of incubation, the cheese pastes were analysed by a panel of at least six experienced tasters. The overall flavour quality (scale ranging from 3 [very poor] to 8 [very good]) and the cheese flavour intensity (scale ranging from 0 [none] to 4 [very strong]) were scored. Overall flavour quality means the general appreciation of the sample, i.e. not focussed on cheese flavour exclusively. The trials were performed 'blind'.

The cheese curds were assessed, after 6 weeks and 13 weeks, as described above. However, only overall flavour quality was scored (scale ranging from 3 [very poor] to 8 [very good]).

2.3.3 Estimation of amino acids. Free amino acids in cheese pastes were determined on a 4151 Alpha Plus amino acid analyser (Pharmacia LKB, Uppsala, Sweden). For this purpose, 9 g of paste were mixed with 18 ml of distilled water and homogenized for 5 min in a stomacher (Seward Stomacher 400 Lab Blender, London, UK). After centrifuging (30,000 *g*, 30 min, 4 °C) defatted supernatants were treated with sulfosalicylic acid (5 %) for removal of proteins and peptides and applied to the amino acid analyser.

2.3.4 Gas chromatography (GC). For the identification of volatile sulphur-containing compounds in cheese paste and cheese curd, the procedure for preparing water-soluble fractions (WSFs) described before [5] was used. Dynamic headspace gas chromatography on WSFs was performed as described previously [1].

3 Results and discussion

3.1 Cheese paste experiments

The results of the sensorical evaluation of the cheese pastes are presented in Table 1.

Table 1. Flavour scores of cheese pastes incubated with methionine and CFE>500 from *Lactococcus lactis* subsp. *cremoris* B78.

Paste	Overall flavour quality[1]	Cheese flavour intensity[2]	Dominant flavours in paste
1 paste + water	5.5	0.5	bland
2 paste + methionine	6.5	1.5	slightly Gouda-cheese like
3 paste + CFE	6.5	2	cheese-like, slightly acid/bland
4 paste + CFE + methionine	7.0	2.5	very cheese-like, broth-like

[1] Scale 3 - 8
[2] Scale 0 - 4

From the results it is clear that, due to the additions made to the cheese pastes, differences in flavour formation occurred. Because viable counts of (contaminating) microflora, e.g. mesophilic lactobacilli, appeared to be low after two weeks of ripening ($<10^5$ cfu/g) of the cheese paste, the additions mentioned in Table 1 account for the perceived flavour formation. The most pronounced effect on flavour was observed during the incubation of cheese paste with both methionine and CFE>500 of *Lactococcus lactis* subsp. *cremoris* B78 (sample 4, Table 1). However, incubation of CFE>500 of *Lactococcus lactis* subsp. *cremoris* B78 without the addition of methionine (sample 3, Table 1) also resulted in the formation of a cheese-like flavour. The observed flavours in pastes 3 and 4 (Table 1) were definitely cheese-like, although, in contrast with the results obtained during incubation experiments in solutions [4], some non-Gouda flavour characteristics, e.g. sweet, were also observed. This was probably due to conversion of substrates other than methionine present in cheese paste. Examples thereof are breakdown of fats or fatty acids and conversion of other amino acids present in the cheese paste (data not shown). The CFE>500 from *Lactococcus lactis* subsp. *cremoris* B78 presumably contains numerous enzymes for breakdown or conversion of various amino acids [1, 3], as well as proteolytic enzymes [8] capable of producing amino acids. In Figure 1 the amounts of free amino acids present in extracts of the cheese pastes containing methionine (paste 2, Table 1) and with CFE plus methionine (paste 4, Table 1), respectively, are shown. The paste incubated with CFE plus methionine contained about twice the amount of free amino acids (due to the action of proteolytic enzymes). A distinct direct contribution to the observed cheese flavour was not expected by this

increase in free amino acids in the cheese pastes during the two-week incubation period [2, 4].

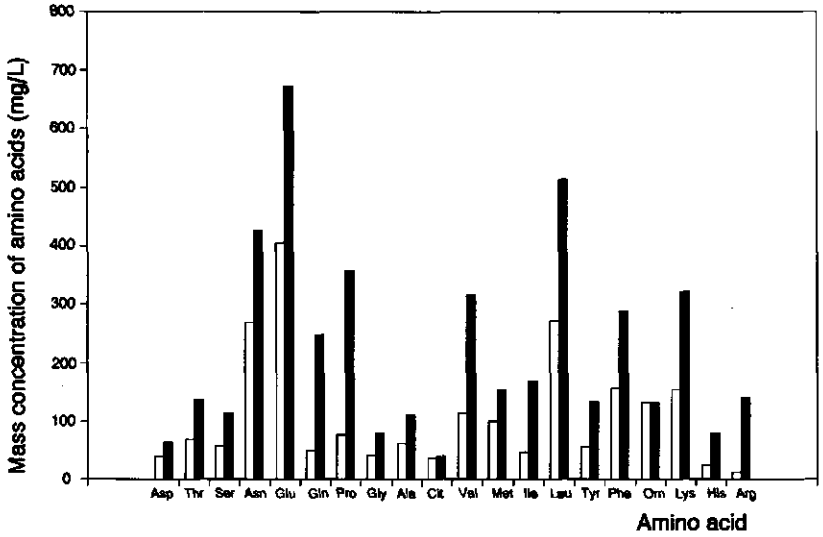

Figure 1. Free amino acids in extracts of cheese pastes.
Paste + methionine: open bars, paste + methionine + CFE: filled bars.

Degradation products of methionine [4], i.e. methanethiol and dimethyldisulphide, were detected in the cheese pastes with CFE>500 (pastes 3 and 4, Table 1) with the aid of GC. Precise quantification however was not possible. Methanethiol and dimethyldisulphide were not detected in the pastes 1 and 2 (Table 1).

3.2 Cheese curd experiments.

The values for fat, salt, pH and moisture, after 14 days of ripening are summarized in Table 2.

Table 2. Compositional data for curds after 14 days of ripening.

Cheese curd	Fat (%)	Moisture (%)	Salt (%)	pH
1 curd + water	29.9	39.6	1.7	5.39
2 curd + methionine	29.4	40.3	2.0	5.32
3 curd + CFE	29.8	40.0	1.8	5.38
4 curd + CFE + methionine	29.9	39.0	1.8	5.42

The values presented are comparable with those of regular Gouda-type cheeses. The consistency of the curds was, however, rather poor. They had a very open texture with many irregular shaped eyes. It seems therefore that the usefulness of the curd system is limited to experimental purposes. Preliminary experiments revealed that approximately 30 - 50 % of the enzymes added and about 15 % of the methionine added were lost in the whey.

The organoleptic evaluation of the cheese curds after 6 weeks of ripening did not reveal significant differences, although the curds with either CFE or CFE + methionine were ranked somewhat higher than the other curds. After 13 weeks of ripening the flavour score for the curd with CFE + methionine was higher as compared with the curds with CFE, methionine or water (Table 3). The cheese-like flavour of this curd was also undoubtedly more intense than that of regular 13-weeks old Gouda cheese.

Table 3. Flavour scores of cheese curds incubated with methionine and CFE from *Lactococcus lactis* subsp. *cremoris* B78 after 13 weeks of ripening.

Cheese curd	Overall flavour quality[1]	Remarks
1 curd + water	5.0	moderately bitter
2 curd + methionine	5.6	moderately bitter
3 curd + CFE	5.8	slightly bitter, sweet
4 curd + CFE + methionine	6.3	slightly bitter, sweet, strongly cheese-like, spicy

[1] Scale 3 - 8, not necessarily Gouda cheese-like

The perceived flavours were somewhat deviant from typical Gouda cheese flavours, probably for the same reason as proposed for the cheese pastes, i.e. the anomalous conversion of various protein and fat derived substrates, such as amino acids and fatty acids, may have occurred in the curds.

The amounts of volatile sulphur compounds in the cheese curds could not be reliably determined, presumably due to the very open texture of the curds.

In summary, from the results of the experiments presented, it may be concluded that enzymes from CFE of *Lactococcus lactis* subsp. *cremoris* B78 are able to convert methionine, and probably also other amino acids, in model systems which closely resemble ripening cheese. Despite the unfavourable conditions [1, 3] in these systems for methionine-degrading enzymes, e.g. low pH and high salt concentration, the conversion of methionine could still be demonstrated.

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Chapter 8

Summary and concluding remarks

Summary and concluding remarks

Flavour is one of the most important attributes of cheese. Cheese flavour is the result of the breakdown of milk protein, fat, lactose and citrate due to enzymes from milk, rennet and microorganisms during production and ripening of cheese [19]. For a large part the development of flavour during the ripening of cheese is determined by the process of proteolysis of caseins. Over the past years proteolysis has been studied very extensively and as a result a wealth of information about this process has been obtained. The ultimate products of proteolysis, amino acids, are prime flavour precursors in cheese and the formation of a "correct" pool of free amino acids is essential for a balanced development of cheese flavour [6, 13, 21]. Amino acids undergo enzymic as well as chemical conversions to essential flavour compounds. However, the formation of cheese flavour compounds by lactic acid bacteria and their enzymes probably is much more important than the formation of flavour compounds by pure chemical reactions, e.g. coupling of carbonyls with amino acids [8].

Until recently, the role of mesophilic starter lactococci, as present in hard-type cheeses (e.g. Gouda), in the process of amino acid degradation was regarded to be limited. Nevertheless, Gouda cheese develops an intense flavour and numerous volatile compounds derived from catabolism of amino acids can be found in this type of cheese.

The purpose of the research described in this thesis was to investigate flavour compounds in the water-soluble fraction of cheese and to elucidate the formation of some of these compounds by lactococcal enzymes.

Chapter 2 describes the production of water-soluble fractions (WSFs) from various types of cheese and the subsequent fractionation of these WSFs by serial ultrafiltration (UF) with membranes of different molecular weight cut-off. As a result fractions containing water-soluble components of various molecular weights were obtained (UF<500 Da, 500<UF<5000 Da and UF>5000 Da). Sensory analysis revealed that low-molecular-weight (<500 Da) compounds were responsible for flavour in WSFs of Cheddar, Gouda, Gouda 20^{+}, Gruyère, Maasdam, Parmesan and Proosdij cheese. The larger peptides and proteins in the UF>500 Da fractions did not contribute directly to the actual flavour of these cheeses, although some flavour attributes, e.g. bitter, are ascribed to larger peptides [20].

The UF<500 Da fractions of the various cheeses include small peptides (probably not larger than tetra peptides), amino acids, fatty acids and further breakdown products of these compounds. In the WSFs of Gruyère, Proosdij and Parmesan cheese large amounts of small peptides and free amino acids were detected, probably due to the action of proteolytic enzymes from thermophilic lactobacilli [1, 11].

The direct contribution of free amino acids to the actual cheese flavour probably is limited [16]. They more likely act as precursors for cheese flavour compounds. Our

taste evaluation results with fractions containing mainly small peptides (<500 Da), obtained from WSF by Sephadex G-10 gel filtration (Gouda cheese) and Sep-Pak C_{18} chromatography (various cheese-types), indicated that such peptides, together with amino acids, must be mainly responsible for basic flavours (e.g. brothy, savoury, sweet). There is not necessarily a relation between cheese flavour and concentration of total free amino acids.

Free fatty acids were detected in relatively high amounts in WSF of Parmesan (butyric acid), Gruyère (butyric and propionic acid) and Maasdam cheese (propionic acid). In these cheeses fatty acids probably play an important role in flavour.

Chapter 3 describes the isolation, identification and possible origin of volatile compounds in the WSFs of 8 hard-type cheeses. The analysis was performed by gas chromatography-mass spectrometry. The cheeses used and the procedure for preparation of WSF (having a distinct cheese-like taste) were the same as applied in Chapter 2.

The volatiles identified belonged to six major groups: fatty acids, esters, aldehydes, alcohols, ketones and sulphur compounds. The flavour attributes of various constituents of each of these groups have been described. Most of the compounds detected were present in the WSF of all eight types of cheese, although their concentrations showed distinct differences. From this it can be concluded that there is not a single compound or class of compounds which is responsible for the full flavour of cheese. Numerous volatile components contribute to the flavour of cheese and our results support the "component balance theory" postulated some 40 years ago [10, 14]. A consequence of this is that it is not possible to describe the flavour of cheese in precise chemical terms.

A considerable portion of the volatiles identified during our study originated from fatty acids (e.g methyl ketones and secondary alcohols) and amino acids (e.g. branched-chain aldehydes and alcohols and sulphur compounds). The breakdown of fatty acids and amino acids is probably governed primarily by enzymic processes and the starter enzymes are a major source of the enzymes involved. Non-starter organisms (e.g. moulds and bacterial surface flora), present in certain types of cheese, naturally also contribute to the formation of flavour compounds.

In hard-type cheeses, such as Gouda and Cheddar, proteolytic enzymes from mesophilic starter lactococci play a crucial role in the formation of free amino acids during ripening. The results of the study described in Chapter 4 indicate that enzymes from mesophilic lactococci are also very important for the formation of (volatile) flavour components from amino acids. In the literature little information is available concerning the significance of amino acid degradation by mesophilic starter bacteria, although the formation of flavour components by certain mesophilic starter strains has been reported [4, 12, 15].

We incubated cell-free extract (CFE), containing all soluble enzymes from *Lactococcus lactis* subsp. *cremoris* B78, a Gouda cheese starter organism, with

methionine, methionine-containing peptides (e.g. fragment α_{s1}-CN(f24-199) from α_{s1}-casein or a mixture of peptides of molecular weight 500 - 5000 Da isolated from Gouda cheese) and fragment α_{s1}-CN(f1-23) from α_{s1}-casein (containing no methionine). These peptides, but also methionine, in itself were tasteless. Sensory analysis showed that a cheese-like flavour only developed during the incubations with methionine and methionine-containing peptides. The formation of relatively large amounts of volatile sulphur compounds, such as dimethyldisulphide and dimethyltrisulphide, from methionine during these incubations could be demonstrated by gas chromatography-mass spectrometry. The use of heat-treated CFE in incubation experiments with methionine did not result in formation of a cheese-like flavour indicating that enzymic activity is necessary. Apart from sulphur compounds other volatiles produced during incubation of CFE with amino acids were identified. An example is 3-methylbutanal originating from leucine.

The experiments in Chapter 4 unquestionably demonstrated that the conversion of methionine by *Lactococcus lactis* subsp. *cremoris* B78 is, at least partially, an enzymic process. Chapters 5 and 6 describe the purification and characterization of enzymes from this organism involved in the conversion of methionine. Two enzymic routes for formation of volatile products from methionine were resolved and in Figure 1 the reactions identified and possible follow-up reactions are shown.

A direct demethiolation reaction of methionine is performed by cystathionine β-lyase. This pyridoxal-5'-phosphate-dependent enzyme is able to catalyse α,β-elimination as well as α,γ-elimination reactions. The latter process (indicated in Figure 1 by dashed lines) results in the production of methanethiol, a very potent flavour compound, from methionine. The physiological role of cystathionine β-lyase is however the conversion of cystathionine during the process of methionine biosynthesis [7]. Although cystathionine β-lyase prefers to catalyze the α,β-elimination reaction (e.g. on lanthionine and cystathionine) α,γ-elimination on methionine does occur under conditions prevailing in ripening cheese, such as a high salt concentration and a low pH. An enzyme similar to cystathionine β-lyase, cystathionine γ-lyase (γ-CTL) was purified from *Lactococcus lactis* subsp. *cremoris* SK11 by Bruinenberg et al. [3]. This enzyme only catalyses the α,γ-elimination of cystathionine and not α,β-elimination. SK11 γ-CTL is also able to convert methionine by α,γ-elimination. However, in contrast to the *Lactococcus lactis* subsp. *cremoris* B78 enzyme, SK11 γ-CTL is unable to degrade L-homoserine and shows relatively high (α,β-elimination activity) toward L-cysteine.

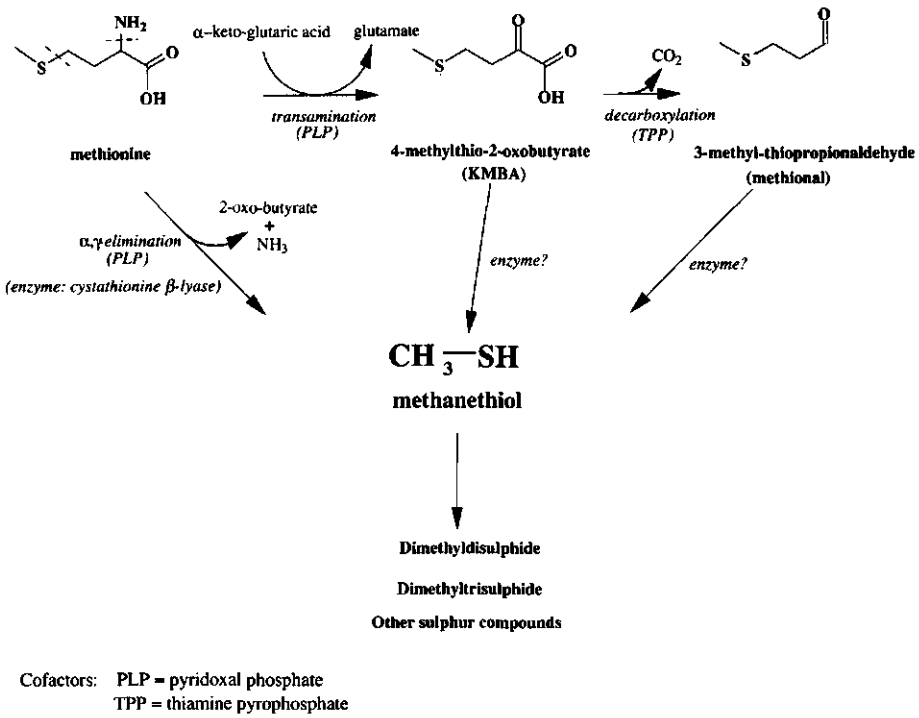

Figure 1. Pathways of the formation of volatile sulphur compounds from methionine by enzymes from *Lactococcus lactis* subsp. *cremoris*.

The transamination of methionine by branched-chain aminotransferases from *Lactococcus lactis* subsp. *cremoris* B78 provided evidence for the existence of an alternative route for the formation of volatile sulphur compounds. This route comprises the conversion of methionine to 4-methylthio-2-ketobutyric acid (KMBA) in the presence of an α-keto acid, e.g. α-ketoglutaric acid (Figure 1). The intermediate KMBA is converted to methanethiol, which can be further converted to other volatile compounds important for cheese flavour e.g. dimethyldisulphide and dimethyltrisulphide. The aminotransferases described in this thesis, AT-A and AT-B, have a broad substrate specificity for both the amino-group donor and the amino-group acceptor. Branched-chain amino acids and α-ketoglutaric acid respectively were the most preferred substrates in this respect. Recently Yvon et al. reported the purification and characterization of an aromatic-amino-acid-converting aminotransferase from *Lactococcus lactis* subsp. *cremoris* NCDO 763 [22]. The enzyme was able to convert

aromatic amino acids but also leucine and methionine and an important role in cheese flavour formation was assumed.

The route of conversion of KMBA is uncertain, however. In eukaryotes the decarboxylation of KMBA by a branched-chain 2-oxo dehydrogenase complex has been reported [9, 18]. The product of decarboxylation of KMBA, i.e. methional (see Figure 1), has been implicated as an important factor in cheese flavour [17, 19]. The route of breakdown of methional however remains uncertain; a direct conversion of KMBA to methanethiol cannot be ruled out (Figure 1). Although up to now we could not establish the formation of methional from KMBA (Figure 1), our experiments have shown that (a) methional is produced during incubation of CFE from *Lactococcus lactis* subsp. *cremoris* B78 with methionine and (b) enzymes from this organism are involved in the conversion of KMBA. The 20-37 % ammonium sulphate fraction obtained from CFE namely facilitated the breakdown of KMBA. Additional experiments showed that thiamine pyrophosphate, a cofactor required for activity of decarboxylases [18], stimulated the enzymic breakdown of KMBA [5].

A transaminative route of degradation of leucine similar to that of methionine (i.e. comprising transamination and decarboxylation) was also suggested by Braun and Olson [2]. The products ultimately produced were 3-methylbutanal and 3-methylbutanol. These compounds, together with analogous compounds produced from isoleucine and valine, are important flavour compounds in various types of cheese. Because the aminotransferases described in Chapter 6 also displayed high activities towards branched-chain amino acids and aldehydes were produced during incubation of CFE with amino acids, it seems that at least two enzymic steps (transamination and decarboxylation) in the catabolism of these amino acids and methionine are mediated by similar enzymes from *Lactococcus lactis* subsp. *cremoris* B78.

In Chapter 7 the results are shown of some preliminary experiments with cheese pastes and curds aimed at testing the role of methionine in combination with starter enzymes under more practical conditions. The observed flavours in pastes to which CFE of *Lactococcus lactis* subsp. *cremoris* B78 had been added were definitely cheese-like and volatile degradation products of methionine were detected with the aid of gas chromatography. The cheese-like flavour of the curds to which CFE and methionine had been added was undoubtedly more intense after 13 weeks than that of regular 13-weeks old Gouda cheese. From the results it is concluded that enzymes in CFE are able to convert methionine in the cheese-like systems, despite the unfavourable conditions e.g. low pH and high salt concentration. This finding is in line with the results of Chapters 5 and 6 which show that both the branched-chain aminotransferases and cystathionine β-lyase are still active under conditions prevailing in cheese.

The results obtained in this study demonstrate that:

(a) The direct contribution of non-volatile compounds formed during the process of cheese ripening, e.g. amino acids and small peptides, to the actual cheese flavour is limited. Volatile compounds formed during the process of cheese ripening undoubtedly are crucial for a proper cheese flavour. These volatiles mainly originate from casein, fat, lactose and citrate.

(b) Amino acid-converting enzymes from starter lactococci, present in hard-type cheeses such as Gouda, play an essential role in the formation of cheese flavours. Currently, the cloning of the genes coding for the enzymes discussed in this thesis is in progress. The construction of genetically modified strains will facilitate further studies to elucidate the importance of these enzymes during cheese ripening.

Although evidence has been provided that in cheese-like systems conversion of methionine to methanethiol and dimethyldisulphide takes place, future studies have to focus on amino acid converting processes in cheese itself. For diversification of cheese flavour not only the conversion of methionine is of importance, but also the conversion of other amino acids present in ripening cheese. Another important aspect is the role of lysis of starter cells during ripening. To assure an adequate interaction between substrates and enzymes lysis of cells, leading to the release of intracellular enzymes into the cheese matrix, is considered to be essential [13]

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Chapter 9

Samenvatting

Samenvatting

Voor de produktie van kaas en andere gefermenteerde zuivelprodukten worden een veelheid aan melkzuurbacteriën en andere micro-organismen gebruikt. Een essentieel gevolg van het fermentatieproces is de toename van de houdbaarheid van de produkten, bijvoorbeeld kaas en yoghurt. Een ander belangrijk aspect is de vorming van een veelal karakteristieke smaak. De vorming van smaak in kaas, het belangrijkste en meest geproduceerde zuivelprodukt, is een gevolg van de omzetting van lactose (melksuiker) en citraat door melkzuurbacteriën, de afbraak van caseïnes (melkeiwitten) en de afbraak van vet. De omzetting van lactose (tot melkzuur) leidt tot verzuring van de melk, terwijl de afbraak van caseïnes door stremsel-enzymen en proteolytische enzymen van melkzuurbacteriën leidt tot het stremmen van de melk en vervolgens tot de vorming van peptiden en aminozuren. Voor de vorming van smaak in kaas zijn met name deze aminozuren essentieel. Aminozuren kunnen tijdens de rijping van kaas namelijk verder omgezet worden tot veelal vluchtige verbindingen die in belangrijke mate de karakteristieke geur en smaak van kaas bepalen. Zowel reacties gekatalyseerd door enzymen uit melkzuurbacteriën en uit andere in of op kaas aanwezige organismen, bijvoorbeeld schimmels, als puur chemische reacties treden op.
Het doel van het in dit proefschrift beschreven onderzoek was tweeledig:

(1) het bepalen van het belang voor geur en smaak van vluchtige en niet-vluchtige verbindingen die worden aangetroffen in de zogenoemde water-oplosbare fractie van diverse kazen (deze fractie heeft een duidelijk herkenbare kaassmaak) en

(2) het in kaart brengen van de vorming van enkele voor smaak belangrijke verbindingen uit aminozuren door enzymen van mesofiele melkzuurlactococcen, zoals onder andere gebruikt tijdens rijping van Goudse kaas.

Hoofdstuk 1 is een algemene inleiding die handelt over de rol van melkzuurbacteriën bij de rijping van kaas. In dit hoofdstuk wordt een overzicht gegeven van de belangrijkste processen die optreden met betrekking tot smaakvorming tijdens de kaasrijping. Met name de proteolyse van caseïnes en de omzetting van aminozuren, gevormd door proteolyse, worden besproken.

Hoofdstuk 2 beschrijft de wijze waarop water-oplosbare fracties werden verkregen uit diverse kazen, alsmede het verder fractioneren, op molecuulgrootte, van deze water-oplosbare fracties met behulp van onder andere ultrafiltratie (UF). De met UF verkregen fracties (UF<500 Da, 500<UF<5000 Da en UF>5000 Da) werden zowel op smaak (en geur) beoordeeld als chemisch geanalyseerd. Het bleek dat verbindingen met een lage molecuulmassa (<500 Da) verantwoordelijk waren voor de smaak van de water-oplosbare fracties uit Cheddar, Goudse, Goudse 20$^+$, Gruyère, Maasdammer, Parmezaanse en Proosdij kaas. Verder bleek dat kleine peptiden en aminozuren die aanwezig zijn in de UF<500 Da fractie niet of slechts weinig direct bijdragen aan de

typische smaak van kaas. Zoals hierboven al werd aangegeven kunnen de aminozuren wel een belangrijke rol spelen als "precursor" voor smaakverbindingen. In de UF<500 Da fractie, en dan vooral in die van Gruyère, Maasdammer en Parmezaanse kaas, werden ook enkele vrije vetzuren aangetroffen die mogelijk een bijdrage kunnen leveren aan de geur en smaak.

In hoofdstuk 3 wordt nogmaals gekeken naar de water-oplosbare fracties van de hierboven genoemde kazen. De aandacht is nu echter speciaal gericht op de aanwezige vluchtige verbindingen. Door gebruik te maken van gaschromatografie-massaspectrometrie (GC-MS) konden zes belangrijke groepen vluchtige verbindingen geïdentificeerd worden: vetzuren, esters, aldehyden, alcoholen, ketonen en zwavelverbindingen. Bijna alle geïdentificeerde verbindingen waren in ieder van de water-oplosbare fracties aanwezig en dus was het niet mogelijk om voor de onderzochte kazen een karakteristieke verbinding of groep van verbindingen aan te wijzen.

Zowel de mogelijke vormingsroutes van verbindingen als hun geur en/of smaak worden beschreven in hoofdstuk 3. Een belangrijk deel van de geïdentificeerde verbindingen wordt gevormd uit vetzuren of aminozuren en enzymatische reacties blijken hierbij een belangrijke rol te spelen.

Hoofdstuk 4 beschrijft modelexperimenten die aantonen dat enzymen van *Lactococcus lactis* subsp. *cremoris* B78 betrokken zijn bij de vorming van geur- en/of smaakverbindingen uit het aminozuur methionine. Door ultrasone behandeling van cellen van *Lactococcus lactis* subsp. *cremoris* B78, een organisme uit Bos zuursel gebruikt voor de produktie van Goudse kaas, werd een zogenoemd celvrij extract (CVE) verkregen met daarin de enzymen van de bacterie. Incubatie van methionine of methionine bevattende peptiden (afkomstig van α_{s1}-caseine of geïsoleerd uit kaas) met CVE onder "kaascondities", dat wil zeggen in aanwezigheid van zout en bij pH 5,4, leverde oplossingen op met een duidelijk kaasachtige geur en smaak. Tevens kon met behulp van GC-MS de vorming van enkele vluchtige zwavelverbindingen, zoals dimethyldisulfide en dimethyltrisulfide, aangetoond worden. Deze vluchtige verbindingen bezitten zeer intense geureigenschappen.

Wanneer bij de incubaties gebruik werd gemaakt van CVE dat een hittebehandeling had ondergaan, werd geen geur- of smaakvorming uit methionine waargenomen. Hieruit valt af te leiden dat na inactivering van enzymen de omzetting van methionine tot aromaverbindingen niet meer plaatsvindt.

De zuivering en karakterisering van enzymen uit *Lactococcus lactis* subsp. *cremoris* B78 die methionine kunnen omzetten tot vluchtige zwavelverbindingen wordt beschreven in de hoofdstukken 5 en 6. We hebben 2 routes waarlangs methaanthiol, zelf een zeer potente aromaverbinding en de "precursor" van verbindingen als dimethyldisulfide en dimethyltrisulfide, uit methionine wordt gevormd, kunnen onderscheiden. Tijdens de eerste route vindt een directe demethiolering plaats van

methionine door het enzym cystathionine β-lyase. De reactie levert naast het al genoemde methaanthiol, 2-oxo-boterzuur en ammoniak op. Cystathionine β-lyase blijkt ook tijdens modelincubaties onder condities zoals die in kaas heersen, dat wil zeggen een hoge zoutconcentratie en een lage pH, in staat methionine om te zetten.

Een tweede route waarlangs methionine omgezet bleek te worden, verliep in meerdere stappen. Als eerste vond transaminering plaats van methionine tot 4-methyl-2-oxoboterzuur (KMBA) door aminozuurtransaminases. We hebben 2 aminozuurtransaminases, AT-A en AT-B, geïdentificeerd die methionine als substraat konden gebruiken. AT-A en AT-B bleken daarnaast ook andere aminozuren, met name leucine, isoleucine en valine, als substraat te kunnen gebruiken.

Het intermediair KMBA wordt enzymatisch verder omgezet tot methaanthiol. De route waarlangs deze omzetting verloopt, is nog niet helemaal duidelijk. Decarboxylering en vorming van methional, en vervolgens methaanthiol, is echter aannemelijk. Net als cystathionine β-lyase blijken de aminozuurtransaminases nog actief te zijn bij hoge zoutconcentratie en een lage pH. De omzetting van aminozuren als leucine en isoleucine levert na decarboxylering respectievelijk 3-methylbutanal en 2-methylbutanal op. Deze beide aldehyden worden in diverse kazen aangetroffen en kunnen ook een bijdrage aan de smaak leveren.

Hoofdstuk 7 beschrijft enkele verkennende experimenten waarbij geprobeerd werd de omzetting van methionine door enzymen van *Lactococcus lactis* subsp. *cremoris* B78 in kaasachtige systemen aan te tonen. Het bleek dat zowel zogenoemde kaaspasta's als wrongel, waaraan methionine en CVE van *Lactococcus lactis* subsp. *cremoris* B78 werden toegevoegd, na enige tijd rijpen een meer intense smaak ontwikkelden dan de overeenkomstige referenties (waaraan dus geen CVE was toegevoegd). In de pasta's met CVE konden bovendien verhoogde concentraties methaanthiol en dimethyldisulfide worden aangetoond.

De bovenstaande resultaten onderschrijven de conclusie dat enzymen van *Lactococcus lactis* subsp. *cremoris* B78 tijdens de rijping uit aminozuren, zoals methionine, verbindingen kunnen vormen die van belang zijn voor de geur en/of smaak van kaas. Verder toont het in dit proefschrift beschreven onderzoek aan dat de directe rol van aminozuren, en peptiden, bij het ontstaan van kaassmaak beperkt is. Juist de vluchtige afbraakprodukten van aminozuren, maar ook van lactose en vet, zijn cruciaal voor de typische smaak en geur van kazen. Voor diversificatie van de kaassmaak is het van groot belang toekomstig onderzoek te richten op dit soort omzettingen en dan vooral op het verloop in de kaas zelf.

Nawoord

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Curriculum vitae

Wim Engels werd geboren op 30 januari 1963 te Tegelen. Na het behalen van het VWO diploma aan het Bouwens van der Boije-College te Helden in 1981, werd in hetzelfde jaar begonnen met de studie scheikunde aan de Katholieke Universiteit te Nijmegen. Het doctoraal examen, met als hoofdvak farmacochemie en als bijvakken biofysische chemie en microbiologie, werd in 1987 behaald. Van november 1987 tot en met december 1988 diende hij als kanonnier der artillerie bij de Koninklijke Landmacht. In juni 1989 trad hij als wetenschappelijk medewerker in tijdelijke dienst bij het Nederlands Instituut voor Zuivelonderzoek (NIZO). Binnen de afdeling Biofysische Chemie werd onderzoek verricht naar smaakvorming in kaas en in 1994 werd besloten dit werk (deels) te vervatten in een proefschrift. Sinds september 1995 is hij als wetenschappelijk medewerker in vaste dienst bij NIZO. Wim is getrouwd met Wies en heeft twee zoons, Guus en Sjoerd.