Bitter flavour in dairy products. II. A review of bitter peptides from caseins: their formation, isolation and identification, structure masking and inhibition

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Review

Bitter flavour in dairy products. II. A review of bitter peptides from caseins: their formation, isolation and identification, structure masking and inhibition

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Summary — Bitterness, a flavour defect liable to be present in dairy products, is due to the accumulation of bitter-tasting peptides. These peptides are rich in hydrophobic amino acids and are formed by the action of proteolytic enzymes on casein. Many studies report the isolation, identification, and characterisation of bitter peptides from cheese and casein hydrolysates, and even their synthesis. This has been done in order to determine the structure of peptides, and also to elucidate the roles of different proteolytic enzyme systems in the development of bitterness, the exact nature of which is still hypothetical. Although bitterness has to be accepted as a necessary consequence of proteolysis, it can be mitigated by masking, removal, or prevention.

bitterness / cheese / casein hydrolysate / peptide / hydrophobicity

Résumé — L'amertume dans les produits laitiers. II. Une revue de la littérature sur la nature des peptides amers issus de la protéolyse des caséines et concernant leur formation, isolement, identification, structure, camouflage et inhibition. *Bien que le défaut d'amertume dans les produits laitiers soit attribuable à la présence, en excès, de certains peptides issus de la protéolyse des caséines et riches en acides aminés hydrophobes, son mécanisme de formation n'a pas encore été complètement élucidé. Dans ce but, plusieurs peptides ont été isolés à partir de fromages ou d'hydrolysats de caséine, identifiés et même synthétisés. Il a alors été possible d'établir certaines relations entre la structure de ces peptides et leur degré d'amertume. Comme le défaut d'amertume est la résultante de la protéolyse et qu'il est susceptible d'être présent dans le produit fini, des techniques telles que le camouflage, la suppression ou l'inhibition de la formation d'une trop grande quantité de peptides amers permettent d'en amoindrir les effets.*

amertume / fromage / hydrolysat de caséine / peptide / hydrophobicité

INTRODUCTION

In a previous paper we have described the possible causes of bitter flavour defect in dairy products, mainly cheese (Lemieux and Simard, 1991). It has also been mentioned that, in cheese varieties other than Cottage, bitter flavour development is associated with proteolysis (Stone and Naff, 1967), which is a basic step in the pathway of flavour development, relying on microbial metabolism of lactic acid bacteria, proteases (chymosin, plasmin, bacterial and mould enzymes) and peptidases (Minamiura *et al,* 1972a; Clegg *et al,* 1974a; Creamer, 1979; Bartels *et al,* 1987b).

α_{s1}-, α_{s2}-, β- and κ-caseins are the main proteins found in cheese. Peptides and amino acids result from their hydrolysis during the cheese manufacturing and ripening processes. These peptides, once isolated and identified, can be evaluated as bitter or not from the hydrophobic property of their amino acid side-chain according to the *Q*-rule proposed by Ney (1971). Detection of bitterness in cheese is possible when the concentration of bitter peptides exceeds a certain threshold level. Indeed, cheese is dynamic in its flavour development, and its bitterness does not result from real off-tasting substances, but from an imbalance which pushes a normal constituent, bitter peptides, to too high a level (Ney, 1971, 1979, 1981).

Although progress has been made to minimise the risk of bitterness in cheese, the exact roles of different proteolytic enzyme systems in the development of bitterness are still hypothetical and the subject of some controversial theories. Among these, one presented by Czulak (1959) proposed that non-bitter starters were capable of degrading bitter peptides released by rennet action to non-bitter products, while Jago (1962) concluded that the differences between bitter and non-bitter strains were due to the inability of bitter strains to hydrolyse the bitter peptides produced by rennet. An experiment conducted by Emmons *et al* (1962a) led them to suggest that strains which produce bitter cheese are deficient in proteolytic enzymes that normally decompose the bitter-tasting peptides. Later on, the hypothesis of Lowrie and Lawrence (1972) postulated that all starter streptococci have the potential to produce bitter cheese. Noomen (1983) has recently reported that interactions between the 3 proteolytic systems of rennet enzymes (predominantly chymosin), starter enzymes, and milk proteinases (plasmin) have an important influence on the content of bitter peptides in cheese without a surface flora, such as Gouda-type and Noordhollandse Meshanger.

Different methods have been developed for the isolation of bitter peptides from various types of cheese. Following their identification, some of these bitter peptides have been shown to be derived from the middle portion of β-casein, and some others from the C-terminal portions of α_{s1}- and β-caseins. However, it could not be established, in any case, that one or more of the peptides isolated formed the main source of the bitter flavour defect in cheese (Visser, 1981).

Synthesis of bitter peptides, analogues and peptides with simplified structure has given rise to studies on structure–taste relationship, providing more information about the mechanism of bitterness. The relative peptide bitterness has been organoleptically determined *via* a panel evaluation of a series of caffeine or quinine sulphate solutions of decreasing concentration.

Although enzymic hydrolysis has the advantage of retaining the nutritional value of the protein, it is often associated with the occurrence of bitter peptides which make the product unpalatable. The role of the

endopeptidases is to split the protein molecule primarily into peptides, while that of exopeptidases is to remove 1 or 2 amino acids from the ends of the peptide molecules. Digestion of casein with the endopeptidase trypsin has thus been shown to result in the formation of one or more bitter peptides for which various amino acid compositions and molecular sizes have been reported (Gordon and Speck, 1965b; Matoba *et al,* 1970; Sullivan and Jago, 1972). Research is also being carried out to find ways to mask or eliminate bitterness off-flavour from these products of hydrolysis. However, before their application, these measures will have to be recognised as safe for the consumer.

This review article on bitter peptides examines their: 1), hydrophobic amino acid side chain; 2), mechanism of formation; 3), isolation and identification; 4), structure; 5), masking and inhibition.

GENERALITIES ABOUT THE PERCEPTION OF BITTER TASTE

Sweet, sour, salty and bitter: the 4 basic taste qualities can be recognised by the human tongue. They are respectively represented by sucrose, hydrochloric acid, sodium chloride and quinine. A fifth taste, attributed to monosodium -L- glutamate and nucleotides, is called "umami", which means deliciousness in Japanese because it has a flavour-enhancing effect (Ikeda, 1909). The back of the tongue is most sensitive to the bitter taste. For a substance to exhibit bitterness, it must be at least slightly soluble in water. The perception or threshold limit is then defined as the lowest concentration of the compound which can be differentiated from water alone. The optimal temperature for the perception of bitterness is 10 °C. Among the basic tastes, bitterness is the only one that could be detected with more sensitivity than umami (Yamaguchi, 1991). The bitter taste response is low but the lingering sensation after a bitter stimulation suggests that the binding of bitter molecules to the bitter receptor is of greater duration than the binding to sugars of the sweet receptor.

Sources of bitterness

It is now accepted that the bitter flavour produced during the enzymic hydrolysis of casein is due to some types of peptide (Fujimaki *et al,* 1970a). The first scientist to report that cheese bitterness resulted from a peptide was Raadsvell (1953), who also initiated the isolation of bitter peptides from casein hydrolysates (Carr *et al,* 1956; Gordon and Speck, 1965b) and from cheese (Harwalkar and Elliott, 1965). As reported by Ney (1979) many other substances, such as amino acids, amines, amides, substituted amides, long chain ketones and some monoglycerides present in cheese might also add to its bitterness. Among proteins, casein produce more bitterness than others and among the different caseins, α_{s1}-casein is regarded as the main source of bitter peptides (Richardson and Creamer, 1973; Adda *et al,* 1982).

History of bitter taste evaluation

In the early sixties, Tanford (1962) calculated the free energies of transfer (in calories) of the amino acid side chains from ethanol to water. Dunhill (1965) then introduced the expression "hydrophobicity" for these values (Guigoz and Solms, 1976). The way for a calculation of bitterness was later opened by Ney (1971) who, introducing Tanford values, published the *Q*-hypothesis, which establishes a semi-quantitative relationship between the amino acid composition of a peptide and its

bitterness. Another hydrophobicity scale for the amino acids has been proposed by Bull and Breese (1974). It is based on the effect of the amino acids on the surface tension of water (the greater the surface tension lowering, the greater the hydrophobicity). Leucine was found to be the most surface active of the amino acids.

According to Ney (1979), no particular amino acid or sequence was needed to impart the bitter taste. However, Japanese workers (*eg*, Kanehisa *et al*, 1984; Shinoda *et al*, 1985a, b; 1986a, b) later proved, by synthesising bitter peptides and several analogues, that the nature of the terminal amino acids and their steric parameters had some influence on the intensity of bitter taste.

Independent of Ney (1971), Matoba and Hata (1972) also proposed that hydrophobic amino acid side chains are responsible for the bitter taste, regardless of the amino acid sequence. Furthermore, they observed that a hydrophobic amino acid exerted the strongest bitterness when both its end were blocked, *eg* by forming peptide linkages. Bitterness was weaker when the amino acid was in a terminal position, and weakest when it was free.

Ney's (1971) hypothesis, establishing the degree of hydrophobicity as the most important predictor of peptide bitterness, has been supported by other workers (Clegg *et al*, 1974a; Schalinatus and Behnke, 1975a, b; Adler-Nissen, 1976; Gardner, 1978; Gatfield, 1981). However, the effect of the position of an amino acid in a peptidic sequence cannot be determined by the method of Ney (1979) (Ishibashi *et al*, 1987a).

A correlation between threshold value and hydrophobicity value for both free amino acids and dipeptides has been established by Wieser and Belitz (1976). Their taste data, in agreement with Matoba and Hata's results (1972) quantitatively confirmed that dipeptides are more bitter than the corresponding free amino acids, and that bitterness intensity is independent of the sequence of both amino acids. Wieser and Belitz (1975) have also found that the threshold for bitter taste decreases with increasing number of hydrophobic side chains (≥ 3) in the peptide, and increases in the presence of hydrophilic side chains parallel to their polarity. This was later corroborated by Lalasidis (1978), who believed the main source of bitterness in enzymic protein hydrolysates to be peptides of more than 3 amino acids.

Among all the studies concerned with bitterness of protein hydrolysates, only Ney's (1971) extrapolated *Q*-rule links bitterness quantitatively to hydrophobicity. This extrapolation of the *Q*-rule to explain the presence or absence of bitterness in protein hydrolysates was proposed only by Ney (1971), and since then widely accepted.

Q-rule

Bitterness of a peptide is caused by the hydrophobic action of its amino acid side chains. An average hydrophobicity, *Q*, is obtained by summing the amino acid side chain hydrophobicities of a peptide and dividing this value by the number of amino acid residues (Ney, 1979, 1981):

$$Q = \frac{\Sigma \Delta f}{n}$$

Where Δf = free energy of transfer of the side chains of the amino acid residues (hydrophobicity; cal.mol^{-1} according to Tanford (table I); n = number of amino acid residues; Q = average hydrophobicity of a peptide.

As summarised in table II peptides with *Q*-values below 1 300 cal.res^{-1} are not bit-

Table I. Free energy of transfer (Δf and ΔF) of the side chains of amino acids, representing their hydrophobicity.
Hydrophobicité des acides aminés, représentée par l'énergie libre de tranfert (Δf et ΔF) de leurs chaînes latérales.

Amino acid	*Abbreviations*[1]		*R groups*[2] *(cal.mol^{-1})*	*Δf-value*[3] *(cal.mol^{-1})*	*ΔF-value*[4]
Non-bitter amino acids[5]					
Glutamine	Gln	Q	2	−100	+ 970
Asparagine	Asn	N	2	− 10	+ 890
Glycine	Gly	G	2	0	+ 810
Serine	Ser	S	2	40	+ 420
Threonine	Thr	T	2	440	+ 290
Histidine	His	H	3	500	+ 690
Aspartic acid	Asp	D	4	540	+ 610
Glutamic acid	Glu	E	4	550	+ 510
Arginine	Arg	R	3	730	+ 690
Alanine	Ala	A	1	730	+ 610
Cysteine	*Cys*	*C*	*2*		*+ 360*
Bitter amino acids[5]					
Methionine	Met	M	1	1300	− 660
Lysine	Lys	K	3	1500	+ 460
Valine	Val	V	1	1690	− 750
Leucine	Leu	L	1	2420	−1650
Proline	Pro	P	1	2620	− 170
Phenylalanine	Phe	F	1	2650	−1520
Tyrosine	Tyr	Y	2	2870	−1430
Isoleucine	Ile	I	1	2970	−1450
Tryptophan	Trp	W	1	3000	−1200

[1] From Lehninger AL (1972) Biochemistry; [2] *R* groups: (1) nonpolar or hydrophobic; (2) polar but uncharged; (3) positively charged (basic amino acids); (4) negatively charged (acidic amino acids) at pH 6.0–7.0, the zone of intracellular pH; [3] Tandford (1962); Ney (1979); [4] the greater the lowering of surface tension, the greater the hydrophobicity (Bull and Breese, 1974); [5] according to table II.

ter, whereas peptides with *Q*-values higher than 1 400 cal.res^{-1} are bitter. If the *Q*-values lie between 1 300 and 1 400 cal.res^{-1}, no prediction can be made about the peptide bitterness. This principle is vallid for molecular weights (MW) up to approximately 6 000 Da; above this limit, peptides with *Q*-values higher than 1 400 cal.res^{-1} are also not bitter.

Although Ney's (1971) semiquantitative rule can be applied to the majority of known isolated or synthetic peptides with defined amino acid composition, chain length and flavour, there are exceptions. Among these, lysine and proline have too high *Q*-values for non-bitter amino acids, the non-bitter peptide: β: 26-28, H-Ile-Asn-Lys-OH with a *Q*-value of 1 670 cal.res^{-1}

Table II. Bitter taste of peptides in relation to their average hydrophobicity *Q* and molecular weight (Ney, 1979).
Amertume des peptides suivant leur hydrophobicité moyenne Q et leur poids moléculaire (Ney, 1979).

Non-bitter taste	
	$Q < 1\ 300$ cal.res^{-1} Molecular weight: 100–10 000 Da (from one amino acid residue to many amino acid residues or protein)
	$Q > 1\ 400$ cal.res^{-1} Molecular weight: 6 000–10 000 Da
Bitter taste	
	$Q > 1\ 400$ cal.res^{-1} Molecular weight: 100–6 000 Da

(Pélissier *et al,* 1974), the dipeptide esters of L-aspartic acid and L-amino malonic acid are sweet peptides (Belitz *et al,* 1979) and also peptides: H-Val-Ala-OH; H-Ala-Ala-Leu-OH and H-Lys-Ala-OH are bitter although their *Q*-values are below 1 300 cal.res^{-1} (Guigoz and Solms, 1976).

Adler-Nissen (1986a, b) has shown that the extrapolated *Q*-rule is unsubstantiated, both theoretically and empirically, and must be abandoned. According to him the *Q*-values of proteins listed by Ney (1971) are considerably overestimated. The cause of bitterness is the presence and concentration of hydrophobic peptides formed by protein hydrolysis; consequently bitterness does not depend only on the average hydrophobicity value of the hydrolysate, as implied by the extrapolated *Q*-rule. Adler-Nissen (1986b) therefore proposed a description of protein hydrolysate bitterness which takes into account the hydrophobicity distribution of peptides. From an earlier comparative study of the bitter taste of enzymic hydrolysates from cow, ewe and goat caseins, β-casein was found to be more hydrophobic than α_{s1}-casein, and both proteins to have an approximately uniform hydrophobicity along the peptide chain (Pélissier and Manchon, 1976; α_{s1}: 1 170 cal.res^{-1}; β:1 330 cal.res^{-1}; Visser, 1977b); however, the C-terminal region of β-casein is known to be very hydrophobic and thus to have an extremely bitter taste (Shinoda *et al,* 1986b). It was then suggested as postulated previously (Ney, 1971; Pélissier *et al,* 1974), not to take into consideration the total hydrophobicity of a protein to determine whether an enzymic hydrolysate has a chance of being bitter or not. In addition, the development of bitterness was shown to be strongly influenced by the nature of the protease used (Pélissier and Manchon, 1976; Belitz and Wieser, 1985). However, earlier work on casein, lactalbumin, soya protein, zein and gliadin, which developed a bitter taste during hydrolysis with several proteinases, led Petritschek *et al* (1972) to conclude that the formation of a bitter taste was mainly dependent on the amino acid composition and sequence of a given protein but not on the hydrolysing enzyme.

Prediction of bitterness

According to the *Q*-rule it is possible to predict the bitterness of any peptide direct-

ly from its amino acid composition and chain length. Furthermore, the risk of obtaining bitter peptides from enzymic hydrolysis of a protein can also be predicted. For example, high *Q*-value proteins such as casein and soya are prone to produce bitter peptides on enzymic hydrolysis, whereas collagen, which has a low *Q*-value, does not produce bitter peptides (Ney, 1979).

Knowing the structure of a compound, it is possible to predict its bitterness threshold. Indeed, the molecular connectivity indices, which are related to the degree of branching (hence the size and shape) of any considered chemical structure, are significantly correlated with published values for the bitterness thresholds of amino acids, peptides and their derivatives (Gardner, 1980).

Investigations of Fukui *et al* (1983) and Otagiri *et al* (1983) on natural and synthetic bitter peptides from casein hydrolysates have led them to suggest that while the *Q*-value is important to overall bitterness, bitterness intensity may not be directly related to the *Q*-value.

It seems that monitoring of the initial rates of proteolysis in stored milk may be efficient in predicting the degree of bitterness and the subsequent shelf-life of ultra-high temperature (UHT) milk sterilised by injection of steam directly into the milk. However, some studies are still necessary to establish sensory scores corresponding to significant bitterness and to ascertain whether these scores are of commercial significance (McKellar *et al*, 1984).

MECHANISM OF BITTER FLAVOUR FORMATION

Since it can limit acceptance of the cheese, the development of bitterness constitutes an economic problem. The complex proteolytic enzyme system involved in the cheese ripening process is presented in figure 1. The characteristic flavour of ripened hard cheese products such as Cheddar and Gouda results from this enzymic system, which may include: 1) the proteinases associated with rennet (chymosin and pepsin); 2) other proteinases

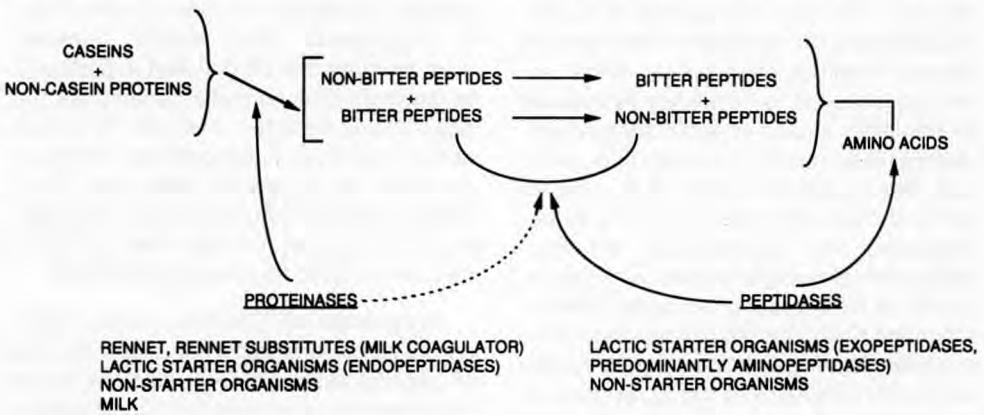


Fig 1. Proteolytic reactions involved in bitter peptides formation in ripened hard cheese products (source: Rouseff, 1990).
Réactions protéolytiques impliquées dans la formation de peptides amers chez un fromage affiné à pâte ferme (source: Rouseff, 1990).

used as the milk coagulant (rennet substitutes); 3) the proteinase/ peptidase system of the starter microorganisms; 4) the proteinase/peptidase system of the non-starter microbial flora; 5) the proteinases endogenous to milk. When the delicate balance of these proteolytic reactions is not optimal, bitter peptides can accumulate to a certain level and give a bitter flavour to cheese. Many studies have tried to elucidate this complicated defect, which is affected by many factors (Lemieux and Simard, 1991), and several hypotheses have been put forward related to the formation of bitter principles in cheese.

It was established early on that the bitter components in cheese reside in the peptide fraction (Kelly, 1932; Raadsveld, 1953), and arise from interactions between bacterial and rennet proteolysis (Dinesen, 1974; Huber and Klostermeyer, 1974). However, the mechanism of bitter peptide formation/degradation in cheese ripening is difficult to elucidate, as witnessed by the conflicting reports in the literature.

A mechanism involving the combined action of rennet and bacterial proteolytic enzymes has been postulated by Czulak (1959) for the development of bitterness in cheese. Peptides released by rennet action are believed to be further hydrolysed by non-bitter strains of lactic streptococci, whereas bitter strains are unable to hydrolyse these peptides, which thus accumulate and cause the bitter taste. He further suggested that accumulation of bitter-tasting peptides might be due to increased activity of rennet at low pH levels. Hence, according to Czulak the starter streptococci do not play any significant part in the development of bitterness (fig 2). In general, the findings of many investigators agree with those of Czulak (Emmons *et al,* 1962b; Jago, 1962; Stadhouders, 1962).

Shorthly thereafter, it was shown that the strains of bacterial starter used for cheesemaking were linked with the bitterness of the final cheese. Emmons *et al* (1960a, b; 1962a) were the first to carry out a systematic investigation on the effect of manufacture conditions on the formation of cheese bitterness, and found the strain of starter organism used to be the main factor responsible. This confirmed the earlier work of Riddet *et al* (1932) and gave birth to a classification of starters as "bitter" and "non-bitter". In accordance with these experiments, the results obtained by Stadhouders (1962) and Czulak and Shimmin (1961) with different starters demonstrated the importance of a specific ability of streptococci to break down rennet peptides. Results obtained by Emmons *et al* (1962a), where bitterness was directly related to the level of trichloroacetic acid (TCA) soluble peptides and inversely related to the level of amino acids in Cheddar cheese, have demonstrated and emphasised the role of the starter organisms in the development of bitter flavour in cheese. The mechanism proposed by Emmons *et al* (1962a, b) involves the combined action of rennet and bacterial proteolytic enzymes, whereby peptides released by rennet action are hydrolysed further by non-bitter strains of lactic streptococci. Bitter strains, however, were presumed to be deficient in proteolytic enzymes that normally decompose the bitter-tasting peptides, and then to be unable to hydrolyse these peptides which accumulate and cause the bitter taste. Their data suggested that non-rennet enzymes, probably bacterial in origin, play an important role in Cheddar cheese proteolysis.

Manufacture of Cheddar cheese under controlled bacteriological conditions and use of gluconic acid lactone in place of starter has helped many workers to suggest that the starter by itself must be partly responsible for the development of cheese flavour (Mabbitt *et al,* 1955; Perry and McGillivray, 1964; Reiter *et al,* 1967); however, other organisms also affect cheese flavour.

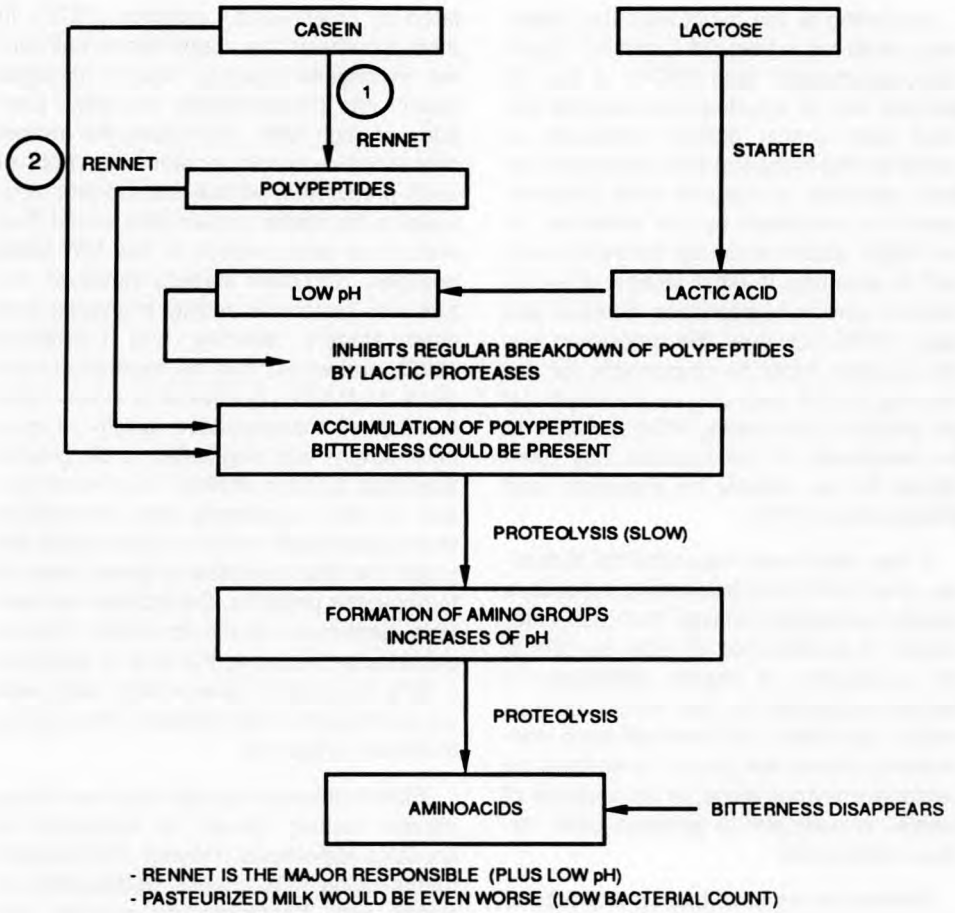


Fig 2. Czulak's hypothesis (source: Furtado, 1984).
Hypothèse de Czulak (source: Furtado, 1984).

Based on their observations that bitter starters, in contrast to non-bitter strains, could form bitter compounds from casein in the absence of rennet, Gordon and Speck (1965a, b) suggested that a modification of Czulak's hypothesis was in order. They attributed their results to a greater proteolytic activity of bitter strains.

Although Czulak's hypothesis (1959) is generally supported by Lawrence and Gilles' data (1969), these authors caution against its complete acceptance, suggesting that some modifications are necessary to account for the role played by fast starters (some of them can produce bitterness on their own or at least supplement that due to rennet) and the controlling influence upon the proteolytic activity of both rennet and starter by salt-in-moisture levels.

According to the hypothesis that bitterness could result from the formation of pyrrolidonecarboxylic acid (PCA) at the N-terminal end of a hydrophobic peptide derived from casein during proteolysis, a model for the formation and degradation of bitter peptides in cultured dairy products would be described by the presence, in non-bitter strains of Group N streptococci, and its absence, in bitter strains, of a pyrrolidone carboxylyl peptidase (Sullivan and Jago, 1970). However, the suggestion that this enzyme might be responsible for debittering rennet peptides by hydrolysis of the peptide bond joining PCA residues to the remainder of the peptides has been shown to be unlikely by Exterkate and Stadhouders (1971).

It has also been suggested by Klimovsky *et al* (1970) that bitter starters possess greater proteolytic activity than non-bitter strains. A positive role for bitter starters in the production of cheese bitterness is equally suggested by the results of Harwalkar and Seitz (1971), which have conclusively shown that casein proteolysis by bacterial enzymes alone, in the absence of rennin, is sufficient to produce bitter flavour components.

Recent results have been interpreted to show that all starter strains are potentially able to contribute directly to the formation of bitter-flavoured components in cheese. Indeed, intracellular proteinase and peptidase activities are present in most of the common bacteria used in cheese-making (*eg Lactococcus lactis* subsp *lactis, Lactococcus lactis* subsp *cremoris*; Castberg and Morris, 1976). The response of each strain to manufacturing conditions such as cooking temperature determines the likelihood of bitterness development (Lawrence *et al,* 1972; Lowrie *et al,* 1972; Martley and Lawrence, 1972).

Following these observations, an alternative hypothesis to Czulak's was formulated by Lowrie and Lawrence (1972). In their hypothesis the major role of calf rennet in cheese ripening was to degrade casein into predominantly non-bitter peptides of high MW. Increasing the rennet concentration would increase the pool of such precursors, whose subsequent degradation by starter proteinases would thus lead to an accumulation of low MW bitter peptides. Non-bitter starters, however, exhibit less proteolytic activity in cheese than bitter starters (Martley and Lawrence, 1972), and would thus be expected to degrade high MW peptides at a slower rate. In addition, the peptidase activity of non-bitter strains was suggested to be greater than that of bitter strains. To a lesser extent it was considered that intracellular starter peptidase could in some cases degrade the bitter peptides to amino acids or to non-bitter peptides. Contribution by bacterial peptidases to the formation of bitter peptides by reducing the size of peptides initially too large to give a bitter taste was equally thought to be of lesser importance, as shown in figure 3.

Effects of bacteriophage infection during cheese making cannot be explained by Czulak's hypothesis. Results from experiments using milk cheese deliberately infected with bacteriophage provide evidence that the starter streptococci play a direct role in the development of cheese bitter flavour. The presence of bacteriophage prevented bitterness in cheeses made under conditions that should have given high residual rennet, negating the contention that residual rennet in cheese was important in bitter flavour production in this case (Stadhouders, 1974; Stadhouders and Hup, 1975). However, a simple explanation for the role of bacteriophage in preventing excessive development of bitter flavour is put forward with Lowrie and Lawrence's hypothesis. Thus, if starter strain enzymes give rise to bitter peptides, reduction of the starter population by massive

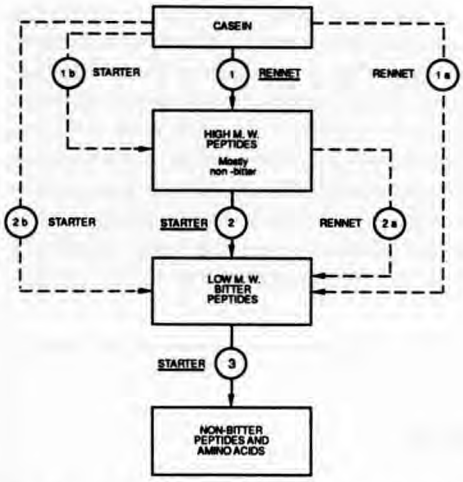


Fig 3. Lowrie and Lawrence's hypothesis (source: Lowrie and Lawrence, 1972).
Hypothèse de Lowrie et Lawrence (source: Lowrie et Lawrence, 1972).

phage attack in the last stages of cheese manufacture should reduce the potential to form bitter compounds (Lowrie, 1977).

While Lowrie and Lawrence's (1972) hypothesis has received considerable general support (Fox and Walley, 1971; Jago, 1974; Sullivan *et al,* 1974; O'Keeffe *et al,* 1975), it has never been claimed to apply to bitterness development in cheese varieties other than Cheddar, although it does appear to explain bitterness formation in Camembert cheese. It should be noted, however, that the influence of rennet on the intensity of bitterness may be less for Camembert than for Cheddar cheeses. Proteolytic activity of the surface mould flora *(Penicillium caseicolum)* of Camembert cheese may play a more important role in providing precursors in themselves not bitter, which are further degraded to bitter peptides by starter proteinases (Martley, 1975a, b).

In support of Lowrie and Lawrence's (1972) hypothesis, according to which bitterness results from starter protease action on rennin produced peptides, the results obtained by Richardson and Creamer (1973) have suggested the likely precursor to be α_{s1}-casein.

In the meantime, studies on proteolysis rates during ripening have led Green and Foster (1974) to conclude that: 1) starter enzymes and rennet are synergistic in their action on casein and its breakdown products; and 2) starter enzymes could be responsible for at least half the cheese proteolysis.

Findings since the publication of the above-mentioned hypotheses on the existence of bitter flavour in cheese have led Stadhouders and Hup (1975) to publish a tentative scheme for the formation and breakdown of bitter peptides in Gouda cheese, paying special attention to the amount of rennet retained in the cheese. They suggested that the synergistic effect of bitter peptides produced by rennet and those produced by the specific proteinase system of bitter starters may be important for the development of bitter flavour in Gouda cheese. In their opinion, cheese may have a bitter flavour when a disproportion exists between the production of bitter peptides from casein by rennet and the breakdown of bitter compounds by the proteolytic enzymes of the starter bacteria, resulting in a concentration of bitter peptides above their threshold value(s); a non-bitter starter also has the ability to degrade bitter peptides. The degrading enzymes of non-bitter and bitter strains were both found to be active at neutral pH; however, those of bitter strains are less active at pH 5.0 (Sullivan *et al,* 1973). Formation and breakdown are affected and influenced as shown in figure 4. Although not proven, this concept is supported by a large number of experimental results.

Although the hypothesis of Lowrie and Lawrence (1972) was developed for Ched-

dar cheese, it is also applicable to Camembert cheese (Martley, 1975b); however, this theory does not seem to fit the results on bitterness in Gouda cheese, which has a composition and ripening conditions which are different to those of Cheddar cheese. High cooking temperatures and early salt addition to the curd in Cheddar manufacture possibly influence the proteolytic properties of starter bacteria.

Aseptically made cheeses, in which the action of starter bacteria and rennet could be separated, were used by Visser (1977a, b) to study various contributions of these enzyme systems. He was able to show that rennet and bitter starter bacteria were independently able to produce bitterness in Gouda cheese. However, the starter bacteria appeared to do this far more specifically than did rennet. According to his results,

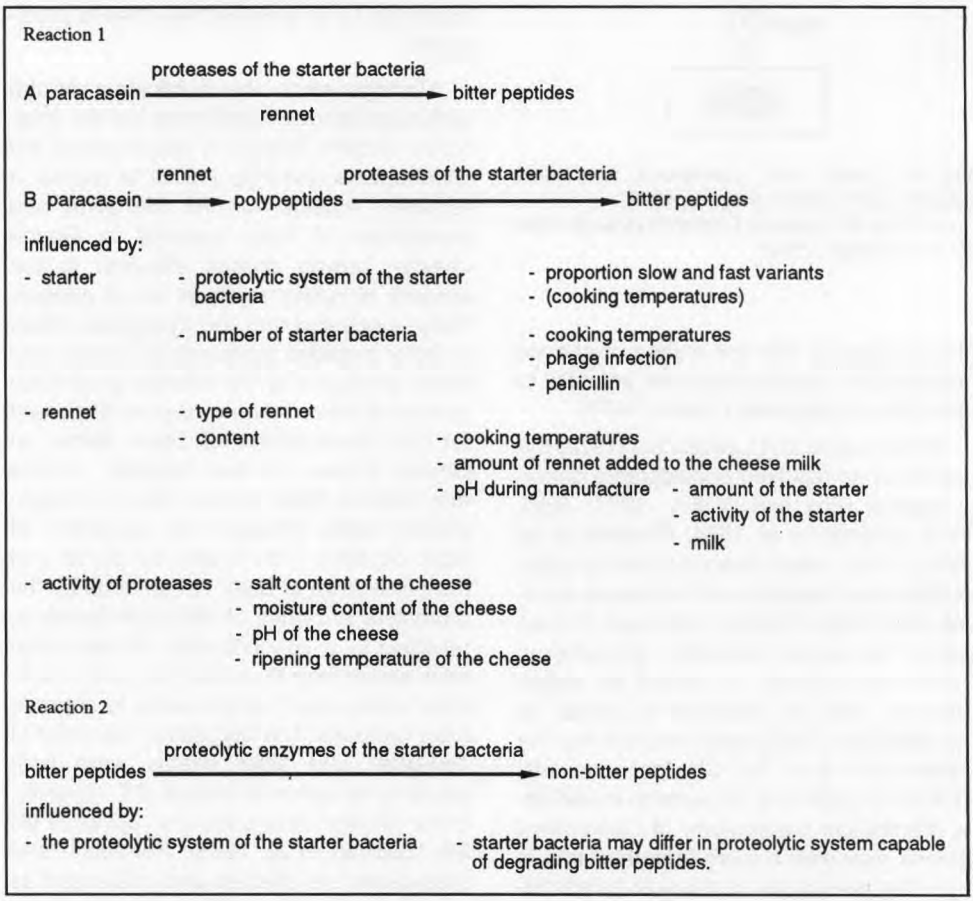


Fig 4. Tentative scheme for the formation and breakdown of bitter peptides in cheese (source: Stadhouders and Hup, 1975).
Schéma suggéré pour la formation et la dégradation des peptides amers dans le fromage (source: Stadhouders et Hup, 1975).

bitter peptides can be degraded to a considerable extent by non-bitter starters under the conditions existing in cheese. The greater part of the observed bitterness in normal aseptic cheeses was due to the separate actions of rennet and bitter starter bacteria. According to Visser (1977a, b) a schematic representation for the different actions involved in the bitterness development in Gouda-type cheeses is given in figure 5.

Exterkate's results (1976) concerning the non-unique responsibility of starter streptococci for bitter development in cheese were in agreement with the hypothesis of Lowrie and Lawrence (1972). Although unable to give a general explanation, Exterkate suggested 3 possible explanations for the development of a non-bitter cheese prepared with different types of bitter and non-bitter strains of *Lactococcus lactis* subsp *cremoris*; 1) the starter itself is unable to liberate bitter peptides at a level sufficient to exceed a certain threshold. It is thereby assumed that liberation of bitter peptides by the action of rennet alone does not result in a bitter cheese; 2) bitter peptides liberated by the action of starter proteases and rennet can be degraded efficiently by the same starter; 3) the membrane-bound endo- and exopeptidase system degrades the hydrophobic bitter peptides. The last explanation seemed to Exterkate to be the most logical for the production of a non-bitter cheese.

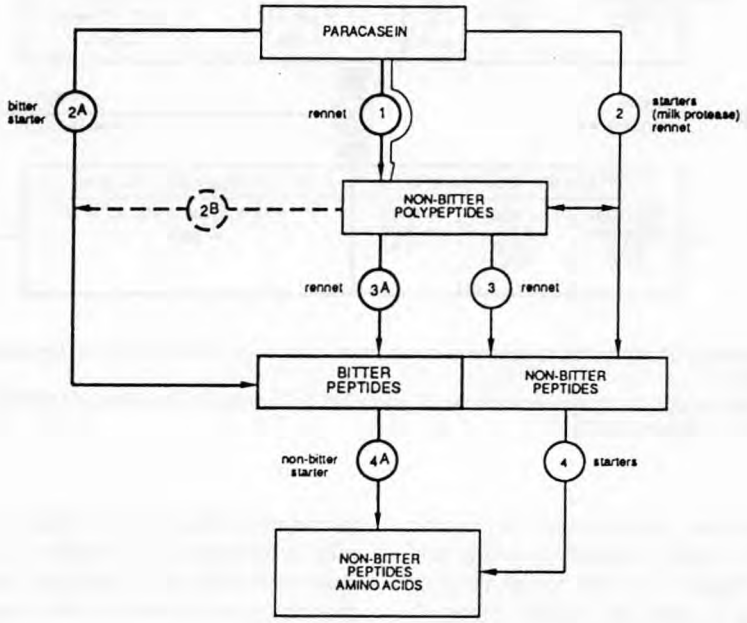


Fig 5. Mechanism for the development of bitterness in Gouda-type cheese. Thin lines: paths of general proteolysis in cheese; thick lines: paths important for bitterness development (source: Visser, 1977a).

Mécanisme d'apparition de l'amertume dans le fromage Gouda. Les lignes fines représentent la voie de la protéolyse en général dans le fromage. Les lignes épaisses représentent les voies importantes pour le développement de l'amertume (source: Visser, 1977a).

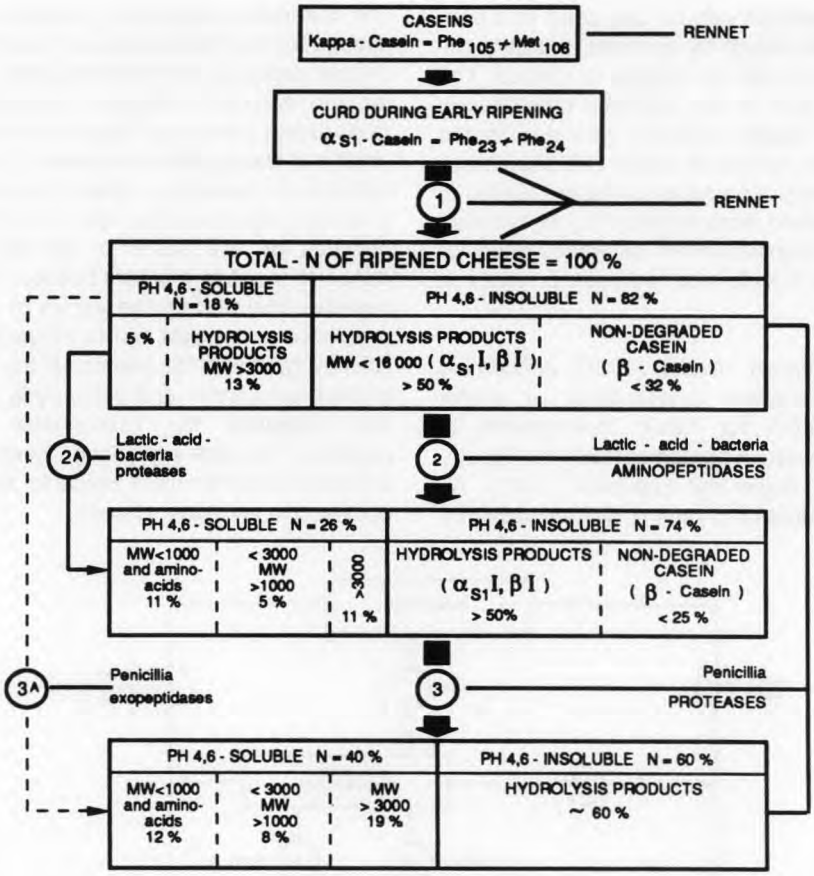


Fig 6. Tentative scheme for the breakdown mechanism of cheese protein (source: Desmazeaud and Gripon, 1977).
Représentation suggérée du mécanisme de dégradation de la protéine (caséine) du fromage (source: Desmazeaud et Gripon, 1977).

The general mechanism of protein breakdown during cheese ripening presented in figure 6 is valid for all types of cheeses up to step (2) + (2A). Steps (3) and (3A) are valid for *Penicillium* cheese. This scheme has been proposed by Desmazeaud and Gripon (1977) from the following observations made with aseptic curds (Cheddar, blue, and cheese with controlled flora or with surface mould) obtained according to a technique of artificial milk acidification by addition of deltagluconic acid lactone: 1) rennet is the first proteolytic agent involved in the overall mechanism of casein breakdown in cheeses; it has a strong and early action on α_{s1}-casein (Gripon *et al,* 1975). Rennet mainly produces peptides of high molecular weight; 3 000 < MW < 16 000 Da (Green and Foster, 1974; Gripon *et al,* 1975) rep-

resenting ~50% of the total nitrogen, and does not apparently induce the release of free amino acids in the curd; thus Gripon *et al* (1975) have found that after 40 days of ripening, low MW peptides (MW < 3 000 Da) constituted only 5% of the total nitrogen content; 2) on the other hand, the action of proteolytic enzymes from lactic acid bacteria leads primarily to the formation of amino acids and short chain peptides, but also to a slight endopeptidase activity; 3) a very active proteolysis is observed in cheeses with surface mould or in blue cheese. Indeed *Penicillium* possesses strong endopeptidase and exopeptidase activities, resulting in the release of large amounts of amino acids; 4) milk proteases induce the breakdown of β-casein.

When too many peptides in a cheese possess the correct characteristics of size and hydrophobicity which are the main criteria for bitter flavour, the cheese is bitter (Creamer, 1979). Ney (1971) has shown that bitterness is related to hydrophobicity, with the more hydrophobic peptides having a more intensely bitter flavour. Only relatively small peptides, because of their solubility, have been found to be bitter (Pélissier *et al*, 1974; Visser *et al*, 1975; Wieser and Belitz, 1976). A diagrammatic representation of the origin of bitterness in Cheddar cheese, from Creamer's results, is shown in figure 7; the caseins (average hydrophobicity (Bigelow's parameter): α_{s1}-, [*Q*-value = 1 170 cal.res^{-1}] α_{s2}-, β-, [*Q*-value = 1 330 cal.res^{-1}] and κ-, [*Q*-value = 1 285 cal.res^{-1}]; Gordon and Groves, 1975), with a *Q*-value of about 1 300 cal.res^{-1} are hydrolysed during cheese maturation through a number of intermediate steps to give a range of peptides, some bitter, some not (arrowheads). During this process the initial degradation of

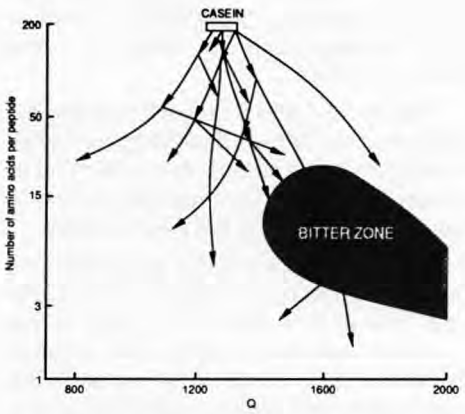


Fig 7. Possible degradation pathway of casein during cheese maturation. *Q* = peptide hydrophobicity, according to Ney (1971) (source: Creamer, 1979).
Processus de dégradation possible de la caséine au cours de la maturation fromagère. Q = hydrophobicité du peptide, d'après Ney (1971) (source: Creamer, 1979).

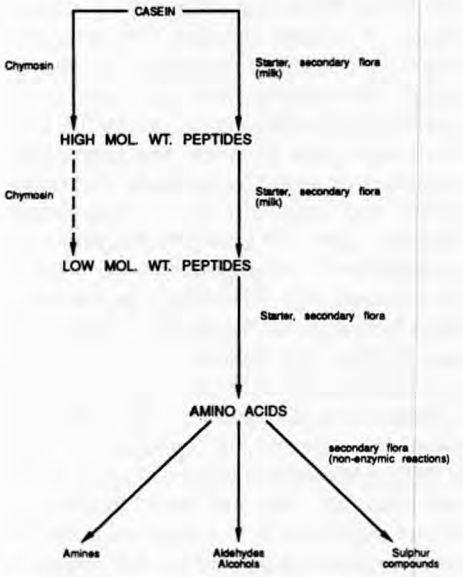
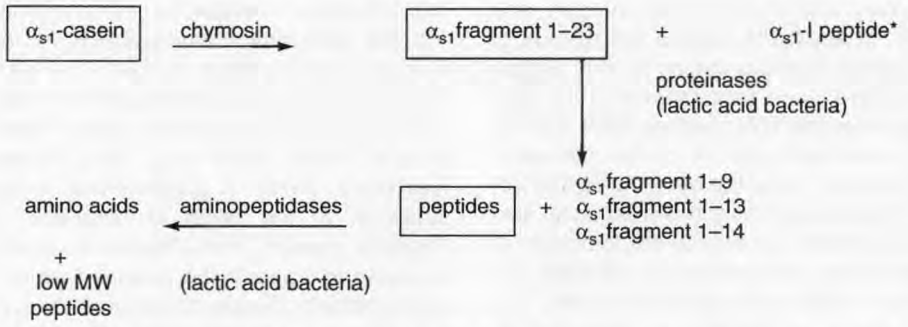


Fig 8. Breakdown of casein during cheese ripening: involvement of proteinases from various sources (source: Law, 1987).
Dégradation de la caséine au cours de l'affinage du fromage: implication de protéinases de différentes sources (source: Law, 1987).



*α_{s1}-I peptide: is composed of residues valine$_{25}$ to tryptophan$_{199}$ or phenylalanine$_{24}$ to tryptophan$_{199}$ (Desmazeaud and Gripon, 1977).

Fig 9. Schematic representation of the degradation of α_{s1}-casein in cheese (source: Law, 1987). *Représentation schématique de la dégradation de la caséine α_{s1} dans le fromage (source: Law, 1987).*

casein is more likely to be caused by the rennet enzyme (chymosin), which is important in the initial degradation of α_{s1}-casein (fig 8), or plasmin (alkaline milk protease) which is possibly responsible for the β-casein proteolysis, whereas later (non-specific) degradation is caused by the bacterial and mould enzymes. The zone of bitterness is bounded by solubility, hydrophobicity and peptide size. Observations made by Cliffe and Law (1990), using a reversedphase high-performance liquid chromatography (RP–HPLC) peptide profiling technique on the water-soluble nitrogen fraction of Cheddar cheese and the removal of bitter taste from rapidly-ripened Cheddar cheese slurries have brought them to support the concept of Law (1987), presented in figure 9, and which demonstrates that the main function of starter peptidase is to further degrade the large peptides produced by the rennet to small peptides and free amino acids.

According to Visser (1981), it has been accepted that enzymes from starter bacteria play a principal role in the further breakdown of cheese protein fragments and thus in the development of cheese flavour.

β-casein hydrolysis by rennet gives rise to the formation of 3 products: 1) fraction β-I, consisting of a mixture of the β-casein arginine$_1$- alanine$_{189}$ and arginine$_1$- leucine$_{192}$ fragments; 2) fraction β-II, composed of arginine$_1$- leucine$_{165}$ and arginine$_1$- glutamine$_{167}$ fragments; 3) fraction β-III, corresponding to rarginine$_1$- leucine$_{139}$ residues .

Peptides β-II and β-III result from the hydrolysis of β-I (Visser and Slangen, 1977); in addition, these authors concluded that β-casein is cleaved by chymosin in only a few definite regions of the protein chain.

Rennet has a strong and early action on α_{s1}-casein. Results of Gripon *et al* (1975) have shown that after 24 h, 40% of the α_{s1}-casein has been broken down, leading to the formation of the α_{s1}-I peptide. Free amino acids and peptides released by proteolytic enzymes such as chymosin and lactic acid bacterial proteases in cheese have been shown to contribute to the formation of cheese flavour (Guigoz and Solms, 1974; Hamilton *et al,* 1974; Huber and Klostermeyer, 1974; Biede and Hammond, 1979). Rennet is known to preferentially attack at the carboxylic side of pheny-

Table III. Bitter peptides isolated from cheese.
Peptides amers isolés du fromage.

Sample	*Origin*	*Peptide sequence*	*Hydrophobicity*[1] *Q cal.res*$^{-1}$	*Ref*
Cheddar cheese	α_{s1}: 14–17	H-Glu-Val-Leu-Asn-OH	1162.5[2]	Hodges *et al,* 1972 Richardson and Creamer, 1973 Hamilton *et al,* 1974
	α_{s1}: 17–21	H-Asn-Glu-Asn-Leu-Leu-OH	1074.0[2]	Hodges *et al,* 1972; Richardson and Creamer, 1973; Hamilton *et al,* 1974
	α_{s1}: 26–32	H-Ala-Pro-Phe-Pro-Glu-Val-Phe-OH	1930.0	Richardson and Creamer, 1973
	α_{s1}: 26–33	H-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-OH	1688.75	Hodges *et al,* 1972; Hamilton *et al,* 1974
Alpkäse (Swiss mountain cheese)	C-terminal α_{s1}: 198–199	H-Leu-Trp-OH	2710.0	Guigoz and Solms, 1974
Cheddar cheese	β: 46–67[3]	H-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-OH	1580.45	Richardson and Creamer, 1973 Hamilton *et al,* 1974
	β: 46–84	H-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr-Pro-Val-Val-Val-OH	1508.5	Hamilton *et al,* 1974
Butterkäse	β:61–69	H-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-OH	1792.2	Huber and Klostermeyer, 1974
Gouda	β: 84–89	H-Val-Pro-Pro-Phe-Leu-Gln-OH	1983.3	Visser *et al,* 1983b
	β: 193–207	H-Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-OH	1686.7	Visser *et al,* 1983b,c
	and/or β: 193–208	H-Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-OH	1766.9	Visser *et al,* 1983b, c
	β: 193–209	H-Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH	1762.4	Visser *et al,* 1983b, c

[1] according to Ney, 1979; [2] $Q < 1\ 300$ cal.res^{-1} and bitter taste; [3] this peptide lost its bitterness by ready cyclisation of its N-terminal glutamine to pyroglutamic acid at neutral pH and ionic strength of 0.1 or less.

lalanine and leucine residues; thus, accoring to Adda *et al* (1982) bitter peptides obtained from casein should contain more phenylalanine and leucine than others. While α_{s1}-casein has nearly disappeared after 20 days of ripening, β-casein, being more resistant, has been shown to be still present in 2/3 of its initial amount after 40 days of ripening. Thus the major part of intact caseins is represented by this unaltered protein (Gripon *et al,* 1975). Since α_{s1}-casein is recognised as always producing more bitterness than β-casein, the relatively small amount of α_{s1}-casein in ewe's or goat's milk could explain why ewe or goat milk cheeses are usually less bitter than those made from cow's milk (Pélissier and Manchon, 1976). Bitter peptides isolated from cheese and listed in table III originated from both α_{s1} fragment 1-23 and α_{s1}-I peptide.

In brief, bitter peptides appear to be produced from casein by the action of rennet or chymosin as well of proteinases from the cell-wall of certain starter bacteria (Visser *et al,* 1983a, b). Degradation of bitter peptides occurs by the action of proteolytic enzymes from the cytoplasmic membrane of bacterial cells, either in concert with enzymes from the cytoplasm or without their aid. In this way, the cell wall proteinases from *Lactococcus lactis* subsp *lactis* NCDO 763 and from *Lactococcus lactis* subsp *cremoris* HP (P_I-type) have been found to be able, as is chymosin, to specifically cleave peptide bonds in the most hydrophobic part of β-casein (Monnet *et al,* 1986; Visser *et al,* 1988). The initial sites of cleavage are thus mainly located in the C-terminal 53-residue region of the β-casein molecule. Moreover, Reid *et al* (1991) have also observed that the initial hydrolysis of the α_{s1}-casein molecule by the proteinase from *Lactococcus lactis* subsp *cremoris* SK 11(P_{III}-type) occurred principally at sites lying in the C-terminal part of the molecule. Other peptides, although in smaller amounts, were from the N-terminal and the central regions of the α_{s1}-casein. However, they could not identify a clear consensus sequence of amino acid residues surrounding the cleavage sites. The presence of salt strongly influences the relative rate of formation and degradation of bitter peptides by starter cells. As shown by Visser *et al* (1983a),

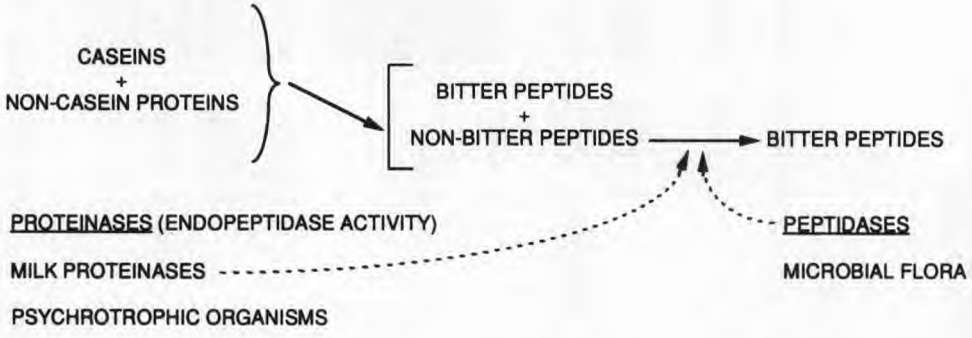


Fig 10. Proteolytic reactions involved in bitter peptide formation in ultra-high temperature (UHT) processed milk products (source: Rouseff, 1990).
Réactions protéolytiques impliquées dans la formation de peptides amers chez les produits laitiers pasteurisés à ultra-haute température (UHT) (source: Rouseff, 1990).

salt mainly seems to prevent at least part of the bitter peptides from penetrating the cell wall and/or the membrane, and thus from becoming accessible to attack by the peptidase system situated near or in the membrane.

Although it has been extensively investigated for ripened hard cheese products, the problem of bitter taste is also associated with fresh market dairy products, yoghurt and other cultured products (*ie* buttermilk, sour cream) and certain ultra-high temperature (UHT) processed milk and cream which may contain heat stable enzymes (Lemieux and Simard, 1991). According to McKellar (1981), when compared to conventionally pasteurised milk, UHT milk seems to be a more suitable substrate for proteolysis and for bitterness development. The complex proteolytic system involved in the production of bitter peptides in UHT processed milk products is presented in the figure 10 (Rouseff, 1990). While a sequential proteolysis involved in the cheese ripening process would give rise to bitter peptide formation (fig 1), it would result directly, in the UHT processed milk products, from the endopeptidase activity originating from the psychrotrophic flora of raw milk. However, the role of microbial peptidases in bitterness of UHT processed milk products has not yet been investigated.

ISOLATION AND IDENTIFICATION OF BITTER PEPTIDES

To elucidate the role of peptides and amino acids and their mechanism of formation in cheese, it is necessary to determine their structure. For this purpose some peptides contributing to the bitter taste have been isolated from cheese and their structures have also been characterised (Guigoz and Solms, 1974; Huber and Klostermeyer, 1974; Visser *et al,* 1983b). Results obtained by Kaminogawa *et al* (1986) and Pham and Nakai (1984) have proposed the reversed-phase high-performance liquid chromatography (RP-HPLC) of the water soluble fraction of cheese as a potential tool for a more objective evaluation of ripening of Cheddar cheese.

Methods for the isolation of bitter peptides from bitter and non-bitter cheeses or from casein hydrolysates

Due to their relatively large content of amino acids with hydrophobic side chains, bitter peptides are lipid-soluble and extractable with organic solvents (Raadsveld, 1953; Sullivan and Jago, 1970). Methods commonly used for isolation of bitter materials from cheese may lead to considerable loss of bitterness where glutamine peptides are involved, as they frequently require the use of solutions of low ionic strength. In neutral solutions of ionic strength 0.1/ or less, the glutamine cyclises readily to pyrrolidone-carboxylic acid, a change accompanied by a loss of bitter taste. Since the rapidity of cyclisation is greatly reduced at NaCl concentrations of 0.2 mol.l^{-1}, it follows that high salt concentrations in cheese actually preserve the peptide in its most bitter form (Hamilton *et al,* 1974).

Several methods are available for extraction of peptides (water soluble nitrogen) from cheese or protein hydrolysates: 5% NaCl for young cheese; buffers at pH 4.6 and gel electrophoresis for young and medium-aged cheeses (less than 6 months old); TCA (2% or 12%), alcohol (70%) and picric acid (0.85%) for mature cheeses (Reville and Fox, 1978). Amongst them precipitation with 70% ethanol and

exhaustive dialysis are recommended if the extract is to be characterised by electrophoresis or chromatography (Kuchroo and Fox, 1983). Further separation and isolation of peptides can be performed by RP–HPLC (Abu-Tarboush *et al*, 1989).

Chromatography on silica gel using an organic solvent such as propanol as a part of the eluent, provides a suitable means of purification of bitter fractions from caseins, and is also very promising for the fractionation of cheese bitter peptides. This isolation technique may be applied in combination with other methods in order to achieve complete purification of bitter peptides from cheese and other products (Gordon and Speck, 1965b; Minamiura *et al*, 1972b; Stadhouders, 1974; Visser *et al*, 1975).

Bitter peptides have been isolated from enzymic casein hydrolysates and from Butterkäse, Cheddar and Gouda cheeses by means of chromatographic techniques using Sephadex G-25 and G-50 (Hettinga and Parmelee, 1965; Schalinatus and Behnke, 1975a; Edwards and Kosikowski, 1983; Sohn and Lee, 1988) and subsequent ion-exchange chromatography on highly acid resin (Huber and Klostermeyer, 1974) or reversed-phase chromatography on a C_{18} (octadecyl silica) column. Bitter peptides from the activated bitter Cheddar cheese contained relatively large amounts of aliphatic, acidic, and hydroxy amino acids, but small amounts of basic and aromatic amino acids (Edwards and Kosikowski, 1983). Relatively large amounts of glutamic acid, proline, leucine, and valine, and a fairly consistent ratio of aliphatic to acidic amino acids were the factors common to most fractions isolated from Gouda cheese (Hettinga and Parmelee, 1965). It has been possible to isolate a pure nonapeptide from 2-dimensional thin-layer chromatograms of the most bitter fraction of Butterkäse cheese (Huber and Klostermeyer, 1974).

Gel filtration chromatography (using Sephadex G-25) of an alcoholic extract of bitter Cheddar cheese has shown that the most bitter fraction occurs in the molecular weight range of 2 000–4 000 Da (Richardson and Creamer, 1970) or 2 000–2 500 Da (Richardson and Creamer, 1973). The bitter component fraction of a casein tryptic hydrolysate has a molecular weight of under 1 000 Da (Matoba *et al*, 1970). Ion-exchange chromatography of these bitter fractions on Dowex 50W-X_4 resin has enabled many peptides to be isolated from the bitter extract.

Bitter-tasting chloroform-methanol extracts (CME) have been obtained from both non-bitter and bitter Cheddar cheeses (Harwalkar and Elliott, 1965; Harwalkar, 1967; Harwalkar and Elliott, 1971; Hamilton *et al*, 1974). Gel filtration chromatography of CME from both cheeses was carried out on a Sephadex G-25 (or on a Sephadex LH-20 (Visser, 1977c; Visser *et al*, 1983b) for a bitter Gouda cheese) column into major fractions which were further resolved by paper and thin-layer chromatography and high-voltage paper electrophoresis, or by high-performance liquid chromatography (HPLC) on a reversed-phase type column (Champion and Stanley, 1982).

Action of proteases and peptidases on caseins during cheese ripening has the potential to produce a myriad of peptides. Recently, Cliffe *et al* (1989) have proposed a procedure that may be suitable for carrying out time course profile studies of peptides produced during the accelerated ripening of Cheddar cheese. Following its isolation by a combination of extraction with water, methanol precipitation, removal of lipid with hexane, and permeation chromatography on Sephadex G-25, the water-soluble nitrogen fraction from Cheddar cheese was then fractionated using reversed-phase fast protein liquid chromatography (FPLC). However, they still have to work on their technique since the resultant

FPLC-chromatogram obtained with a water-methanol eluent was relatively featureless when compared to that obtained by Champion and Stanley (1982). Indeed, the latter have obtained about 71 peaks when the bitter extract from Cheddar cheese coagulated with chicken pepsin was rechromatographed, following gel filtration chromatography on Sephadex G-50, on a reversed-phase HPLC column using a linear gradient from 0 to 90% methanol in water. Champion and Stanley were then able to show that the average molecular weight of the bitter fraction was 190 Da by using gel filtration and ultracentrifugation of HPLC fractions. The bitter fraction obtained from accelerated ripening Cheddar cheese, and separated by size-exclusion HPLC (Lemieux *et al,* 1989), could be further fractionated into its different peptides according to the method followed by Lemieux and Amiot (1990).

Identification of bitter peptides isolated from cheese

A list of characterised bitter peptides isolated from cheese is given in table III. A bitter concentrate, with a polypeptide structure has been isolated from raw milk Gouda cheese (Raadsveld, 1953). Hydrolysis of bitter peptides has shown the presence, in large amounts, of proline, alanine, glutamic acid and valine. Unfortunately, their sequences have not been elucidated. Schalinatus and Behnke (1975a, b) have also isolated bitter peptides from Edam cheese without being able to establish their identity. Zvyagintsev *et al* (1972) have separated and characterised several bitter peptides from different Russian cheese varieties (*eg* Kostroma cheese); all these peptides contained leucine, valine, and phenylalanine residues and the presence of a cyclic N-terminal structure was an important feature.

Bitter peptide fractions found in normal aseptic, aseptic starter-free, aseptic rennet-free and aseptic rennet- and starter-free Gouda cheeses have been shown to have a molecular weight of less than ≈ 1 400 Da (Visser, 1977c). Obviously rennet produces relatively important amounts of small bitter peptides. Indeed, Visser *et al* (1983b) have come to the conclusion that the relatively slow degradation of β-casein by rennet and starter proteinases irrevocably leads to the gradual appearance of bitter fragment 193–209 as the first breakdown peptide. This peptide has also been found to be very resistant to further degradation by chymosin as well as cell wall-associated proteinases from various starters. Thus, unless suitable starter peptidases cause its degradation to non-bitter products, the bitter peptide may slowly accumulate in cheese and start to contribute to a bitter taste in time.

Identification of bitter peptides isolated from casein and casein fractions

Treatment of casein with proteolytic enzymes, rennin (Visser *et al,* 1975), trypsin (Matoba *et al,* 1969; Hill and Van Leeuwen, 1974), and papain (Clegg *et al,* 1974a) has been shown to frequently give rise to digests which are bitter in taste (Hettinga and Parmelee, 1965; Sullivan and Jago, 1972). Proteolytic enzymes with optimal pH on the neutral or alkaline side generally gave more bitterness as compared with enzymes with an acidic optimal pH (Minamiura *et al,* 1972b). Bitterness has been attributed to the presence in milk protein enzymic digests, of peptides composed of unmodified fragments of one or more of the parent caseins (Matoba *et al,* 1970; Sullivan and Jago, 1972).

The same bitter tasting hydrolysate resulted from each α-, β-, and γ- fraction of

Table IV. Bitter peptides isolated from α_{s1}-casein.
Peptides amers isolés de la caséine α_{s1}.

Origin	*Peptide sequence*	*Hydrophobicity*[1] *Q, cal.res*$^{-1}$	*Ref*
α_{s1}: 21–23	H-Leu-Arg-Phe-OH	1933.3	Pélissier *et al,* 1974
α_{s1}: 23–34	H-Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu[2]-Val-Phe-Gly-Lys-OH	1833.3	Matoba *et al,* 1970 Helbig *et al,* 1980
α_{s1}: 23–34	H-Phe-Phe-Val-Ala-Pro-Phe-Pro-Gln[2]-Val-Phe-Gly-Lys-OH (BP-II)	1779.3	Matoba *et al,* 1969, 1970 Belitz and Sparrer, 1971; Mercier *et al,* 1971 Hill and Van Leeuwen, 1974
α_{s1}: 26–33	H-Ala-Pro-Phe-Pro-Gln-Val-Phe-Gly-OH	1607.5	Hamilton *et al,* 1974
α_{s1}: 29–32	H-Pro-Gln-Val-Phe-OH	1715.0	Pélissier *et al,* 1974
α_{s1}: 91–100	H-Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg-OH	1660.0	Hill and Van Leeuwen, 1974
α_{s1}: 92–99	H-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-OH	1625.0	Pélissier *et al,* 1974
α_{s1}: 94–100	H-Tyr-Leu-Glu-Gln-Leu-Leu-Arg-OH	1615.7	Hamilton *et al,* 1974
α_{s1}: 95–99	H-Leu-Glu-Gln-Leu-Leu-OH	1542.0	Mercier *et al,* 1971
α_{s1}: 99–101	H-Leu-Arg-Leu-OH	1856.7	Pélissier *et al,* 1974
α_{s1}: 143–149	H-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu-OH	2101.4	Pélissier *et al,* 1974
α_{s1}: 145–150	H-Phe-Tyr-Pro-Glu-Leu-Phe-OH	2293.3	Belitz and Sparrer, 1971
α_{s1}: 145–151	H-Phe-Tyr-Pro-Glu-Leu-Phe-Arg-OH	2070.0	Mercier *et al,* 1972; Hill and Van Leeuwen, 1974
α_{s1}: 167–179	H-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-OH	1304.6	Pélissier *et al,* 1974
α_{s1}: 198–199	H-Leu-Trp-OH	2710.0	Hill and Van Leeuwen, 1974
Mixture of α_{s1}:	The mixture is bitter		
14–17	H-Glu-Val-Leu-Asn-OH	1162.5	Matoba *et al,* 1970; Hodges *et al,* 1972
17–21	H-Asn-Glu-Asn-Leu-Leu-OH	1074.0	Richardson and Creamer, 1973
26–33	H-Ala-Pro-Phe-Pro-Gln[2]-Val-Phe-Gly-OH	1607.5	
26–33	H-Ala-Pro-Phe-Pro-Glu[2]-Val-Phe-Gly-OH	1688.75	
α_{s1}-casein[3]	[Leu-Trp-Leu-Trp] or cyclo (Trp-Leu-Trp-Leu), [cyclo (Leu-Trp)]*	2710.0	Minamiura *et al,* 1972a Fukui *et al,* 1983; *Kanehisa *et al,* 1984
α_{s1}-casein[4] (145–148)	H-Phe-Tyr-Pro-Glu-OH	2172.5	Belitz and Sparrer, 1971
α_{s1}-casein[4] (149–150)	H-Leu-Phe-OH	2535.0	Belitz and Sparrer, 1971

[1] According to Ney, 1979; [2] residue 30 in the α_{s1}-casein is shown as glutamine by Richardson and Creamer (1973), but is considered to be a glutamic acid residue by Matoba *et al* (1970) and Hodges *et al* (1972). However, Nagao *et al* (1984), by characterising bovine caseins at mRNA levels by cloning cDNAs have assigned glutamate instead of glutamine for position 30; [3] possibly from the C-terminal sequence of α_{s1}-casein (Mercier *et al,* 1971); [4] degradation of α_{s1}: 145–150 with the action of thermolysine.

Table V. Bitter peptides isolated from β-casein.
Peptides amers isolés de la caséine β.

Origin	*Peptide sequence*	*Hydrophobicity*[1] Q, *cal.res*$^{-1}$	*Ref*
β:46–65	H-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-OH	1459.0	Hamilton *et al*, 1974
β:53–79	H-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-OH	1482.2	Clegg *et al*, 1974a
β:61–69	H-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-OH	1792.2	Huber and Klostermeyer, 1974
β: 84–89 (possible)	H-Val-Pro-Pro-Phe-Leu-Glu-OH	2091.7	Visser *et al*, 1983a
β: 103–105	H-Ala-Pro-Lys-OH	1616.7	Pélissier *et al*, 1974
β: 128–139	H-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-Pro-Pro-Leu-Leu-OH	1552.5	Visser *et al*, 1975
β: 167–175	H-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln-OH	1375.6	Monnet *et al*, 1986
β: 176–182	H-Lys-Ala-Val-Pro-Tyr-Pro-Gln-OH	1704.3	Monnet *et al*, 1986
β: 183–193	H-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr-OH	1740.9	Monnet *et al*, 1986
β: 190–192	H-Phe-Leu-Leu-OH	2496.7	Pélissier *et al*, 1974
β: 193–207	H-Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-OH	1686.7	Visser *et al*, 1975
β: 193–208	H-Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-OH	1766.9	Visser *et al*, 1975
β: 193–209[2]	H-Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH	1762.4	Visser *et al*, 1975
β: 194–207	H-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-OH	1602.1	Monnet *et al*, 1986
β: 194–209	PCA[3]-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe Pro-Ile-Ile-Val-OH	1693.2	Gordon and Speck, 1965b Monnet *et al*, 1986
β: 195–209	H-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro Ile-Ile-Val-OH	1812.7	Gordon and Groves, 1975
β: 202–209	H-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH	2031.25	Minamiura *et al*, 1972b
β: 203–208	H-Gly-Pro-Phe-Pro-Ile-Ile-OH	2305.0	Pélissier *et al*, 1974
β: 203–209	H-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH	2217.1	Matoba *et al*, 1970 Pélissier *et al*, 1974 Hashimoto *et al*, 1980
β-casein[4]	H-Gly-Pro-Phe-Pro-Val-Ile-OH (BPI)[5]	2091.7	Matoba *et al*, 1970
β-casein[4]	H-Arg-Gly-Pro-Pro-Phe-Phe-Ile-Val-OH	1991.25	Minamiura *et al*, 1972a, b
β-casein[4]	H-Arg-Gly-Pro-Pro-Phe-Ile-Val-OH (BPI-a)	1897.1	Minamiura *et al*, 1972b

[1] According to Ney, 1979; [2] the conclusion of Pélissier *et al* (1974) that the C-terminal fragment 193–209 of β-casein would be a non-bitter peptide, is not in accordance with the observations of Visser *et al* (1975); [3] PCA: pyrrolidonecarboxylic acid, a derivative formed by cyclization of N-terminal glutamine (Sullivan and Jago, 1970); [4] possibly from the C-terminal sequence of β-casein; this p otein (variant A[2]) has the highest hydrophobicity among caseins (Ribadeau-Dumas *et al*, 1972). No amino sequence of these peptides can be found in the structure of the casein but they are very similar to the C-terminal portion of the β-casein (Hashimoto *et al*, 1980); [5] Hashimoto *et al* (1980) assumed the structure of the natural peptide isolated by Matoba *et al* (1970) to be H-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH and not H-Gly-Pro-Phe-Pro-Val-Ile-OH. However, they could not verify their assumption.

Table VI. Bitter peptides isolated from α_{s2}-casein and casein fractions.
Peptides amers isolés de la caséine α_{s2} et fractions de caséine.

Origin	*Peptide sequence*		*Hydrophobicity[1] Q, cal.res^{-1}*	*Ref*
α_{s2}: 174–181	H-Phe-Ala-Leu-Pro-Gln-Tyr-Leu-Lys-OH	BPIII	1888.75	Matoba *et al,* 1969, 1970
*	H-Leu-Val-Pro-Arg-Tyr-Phe-Gly---	BPI-b		Fukui *et al,* 1983
*	H-Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-Asn-His-OH	BPI-c	1853.0	Minamiura *et al,* 1972b
*	H-Val-Glu-Val-Phe-Ala-Pro-Pro-Phe-OH		1900.0	Belitz and Sparrer, 1971
**	Glu_2-Pro_3,Val_1,Leu_1,Tyr_4, Phe_1		2266.7	Helbig *et al,* 1980
**	Glu_4, Pro_2, Tyr_2, Phe_2		1848.0	Helbig *et al,* 1980

[1] According to Ney, 1979; * bitter peptides which cannot be located as residues of the sequences of α_{s1}- or β-caseins; ** bitter fractions isolated from the butanol extract of the activated carbon used in debittering pronase-hydrolysed skim milk.

the casein complex submitted to a tryptic enzymic hydrolysis. According to Carr *et al* (1956), this polypeptide may have a cyclic portion with a side-chain carrying a C-terminal: H-Leu-Val-Glu-Leu-OH. A bitter peptide isolated from milk cultures of *Lactococcus lactis* subsp *cremoris* has been found to contain 19 amino acid residues (1 arginine, 2 glutamic acid, 2 glycine, 2 isoleucine, 2 leucine, 1 phenylalanine, 5 proline and 4 valine). However, it is not known whether this bitter peptide results from the accumulation of secondary breakdown products, or is the product of a coupling reaction involving peptides and amino acids (Gordon and Speck, 1965b). According to its amino acid composition, this bitter peptide has been reported to be very similar to β-casein fragments 193–209 and 193–207 (and/or 193–208) isolated from Gouda-type cheese by Visser *et al* (1983b). Cell wall proteinases can also hydrolyse caseins and supply peptides. As demonstrated by Monnet *et al* (1986), the cell wall proteinase from *Lactococcus lactis* subsp *lactis* NCDO 763 is able, as is chymosin, to specifically cleave peptide bonds in the most hydrophobic part of β-casein and thus to liberate 5 identified bitter peptides: β: 167–175; β: 176–182; β: 183–193; β: 194–207 and β: 194–209. Bitter peptides isolated from α_{s1}-, β-, α_{s2}- caseins and casein fractions are listed in tables IV, V and VI respectively.

Synthesis of bitter peptide fragments from β- and α_{s1}-caseins

Studies on the structure–taste relationship of synthetic bitter peptides have been carried out chiefly by Japanese workers. Peptides were synthesised by the conventional liquid phase synthetic procedure. In simplifying the structure of peptides they wanted to learn more about the mechanism of bitterness. Synthesised β- and α_{s1}-casein bitter peptide fragments without analogues and peptides with simplified structure (with the exception of H-Arg-Pro-Phe-Phe-OH) are shown in table VII. Thus a strongly bitter heptapeptide, H-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH has been synthesised by Hashimoto *et al* (1980) and was found to be indistinguishable from the natural peptide β: 203–209, isolated from a tryptic hydrolysate of Hammersten casein by means of chromatography, amino acid analyses, and mass spectrometric measurements. In addition, they assumed the structure of the natural peptide isolated by Matoba *et al* (1970) to be β: 203–209 and not BPI (H-Gly-Pro-Phe-Pro-Val-Ile-OH); however, they could not verify their assumption.

In order to elucidate the relationship between the structure of heptapeptide BPI-a (H-Arg-Gly-Pro-Pro-Phe-Ile-Val-OH) and its bitter taste, it has been synthesised, along with many analogues, by many workers (Fukui *et al*, 1983; Miyake *et al*, 1983; Otagiri *et al*, 1983, 1985; Nosho *et al*, 1985). BPI-a is one of the most bitter compounds, comparable with phenylthiourea and quinine, and the results of both taste and circular dichroism measurements (Miyake *et al*, 1983), have shown its bitter taste to be caused by the spatial structure of the molecule conferred by the L-proline residue. The study of Shinoda *et al* (1985b) has confirmed that the number of hydrophobic amino acids alone cannot control the strength to bitterness in peptides, while Nosho *et al* (1985) have observed that, in addition to the hydrophobicity of the molecule, the detailed structure is also important for an increase in bitterness.

Ishibashi *et al* (1988a) have been able to investigate in detail the effect of the amino acid side chains on bitterness. For this purpose, they have synthesised many oligopeptides in which the constituent amino acids are systematically changed in terms of the hydrophobicity of the side chain. They have also studied extensively the sig-

Table VII. Synthesized β- and α_{s1}-casein bitter peptide fragments.
Séquences de peptides amers synthétiques des caséines β et α_{s1}.

Origin	*Peptide sequence*	*Hydrophobicity*[1] Q, *cal.res*$^{-1}$	*TV*[2]	*Rcaf*[3]	*Ref*
β: 60–66	H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH	2335.7		6.3	Kanehisa *et al,* 1984 Shinoda *et al,* 1985a, b; 1986a, b
β: 61–66	H-Pro-Phe-Pro-Gly-Pro-Ile-OH	2246.7	0.44	2.3	Shinoda *et al,* 1985a; 1986a, b
β: 61–67	H-Pro-Phe-Pro-Gly-Pro-Ile-Pro-OH	2300.0	0.25	4.0	Kanehisa *et al,* 1984 Shinoda *et al,* 1985a, b; 1986a, b
β: 82–88	H-Val-Val-Val-Pro-Pro-Phe-Leu-OH	2197.1	0.14	7.1	Shinoda *et al,* 1985a; 1986a
β: 82–90	H-Val-Val-Val-Pro-Pro-Phe-Leu-Gln-Pro-OH	1988.9	0.38	2.6	Shinoda *et al,* 1985a
β: 196–201	H-Pro-Val-Leu-Gly-Pro-Val-OH	1840.0	0.50	2.0	Shinoda *et al,* 1985b; 1986b
β: 196–209	H-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH	1949.3	0.0149	67.0	Shinoda *et al,* 1985b; 1986a, b
β: 200–209	H-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH	2056.0	0.004	250.0	Shinoda *et al,* 1985b; 1986a, b
β: 202–209	H-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH	2031.25	0.004	250.0	Shinoda *et al,* 1985b; 1986a, b
β: 203–209	H-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH	2217.1	0.17–0.34	5.88–2.94	Hashimoto *et al,* 1980
β: 204–209	H-Pro-Phe-Pro-Ile-Ile-Val-OH	2586.7	0.125	8.0	Shinoda *et al,* 1985b
β-casein[4]	H-Arg-Gly-Pro-Pro-Phe-Ile-Val-OH (BPI-a)	1897.1	0.05	20.0	Fukui *et al,* 1983 Miyake *et al,* 1983 Otagiri *et al,* 1983, 1985 Nosho *et al,* 1985
	H-Arg-Pro-Phe-Phe-OH[5]	2162.5	0.04	25.0	Nosho *et al,* 1985
	H-Arg-Gly-Pro-Pro-Phe-Ile-OH	1931.7	0.025	40.0	Shinoda *et al,* 1986a
α_{s1}-casein[6]	cyclo (Leu-Trp)	2710.0	0.063	15.87	Shiba and Nunami, 1974 Fukui *et al,* 1983

[1] According to Ney, 1979; [2] TV = threshold value; [3] Rcaf = the ratio to caffeine; [4] possibly from the C-terminal sequence of β-casein (Ribadeau-Dumas *et al,* 1972); [5] this tetrapeptide produces a bitter taste that corresponds to that of BPIa; however, its molecular weight is only about half that of BPIa; [6] possibly from the C-terminal sequence of α_{s1}-casein (Mercier *et al,* 1971).

nificance of linear and branched side chains in *n*-leucine and *n*-valine on peptide taste, and the influence of structural change to the side chain of the amino acid. Results of these different structural analyses will be resumed in the next section.

STRUCTURE OF BITTER PEPTIDES OR RELATIONSHIP BETWEEN THE CHEMICAL STRUCTURE AND TASTE

Threshold values of bitter substances are much lower than those of sweet, sour and salty substances. Indeed, the values for humans are as follows: strychnine 1.6 $\mu mol.l^{-1}$, picric acid 3.7 $\mu mol.l^{-1}$, quinine 8 $\mu mol.l^{-1}$, nicotine 19 $\mu mol.l^{-1}$, caffeine 700 $\mu mol.l^{-1}$ and theobromine 750 $\mu mol.l^{-1}$. To clarify the physicochemical mechanism of bitter taste sensation Iiyama *et al* (1986) have investigated the effect of bitter substances on a model membrane system of taste reception, and found their type of response to be different from that observed with other taste stimulants.

As mentioned by Kubota and Kubo (1969) a bitter compound must contain at least one "unit" of bitter taste. This "unit" consists of a D*H* group (*eg*, OH, CHO-$COCH_3$, $CHCOOCH_3$ and CHCO groups) and an *A* group (*eg*, carbonyl groups such as CHO, CO, COOH and a double bond such as C=C-O-); these proton–donor and proton–acceptor groups in the molecule must be within a distance of about 1.5 Å. The order of the interorbital *A*-DH distance would then require the binding of bitter molecule to the receptor to be very strong to overcome the tendency for the molecule to adopt an intramolecularly H-bonded state. From their work, Birch and Lee (1976) have concluded that no distinct interorbital distances could yet be established for bitterness but that *A*-DH systems could suffice to explain bitterness in some types of sugar analogues.

A bitter receptor can be excited only if the chemical and steric properties of the molecule match exactly the polar and non-polar contact zones of the receptor. Bitter compounds need only one polar group which may be electrophilic or nucleophilic, and a hydrophobic group; moreover, the hydrophobic contact zone of the receptor has been found to be suitable for molecules up to a dimension of about 10–12 Å by 16–18 Å by 5Å. Changes in molecules caused by introducing an additional functional group or by removing a functional group may not only influence the intensity of taste (bitterness) but also its quality (changes from bitter to sweet or *vice versa*) (Belitz *et al,* 1983).

Knowing that like other biological processes, taste occurs by a substance and receptor interaction, Shinoda and Okai (1985) have proposed a model for the bitter taste receptor of the gustation cell. As seen in figure 11, 2 functional groups are necessary to produce bitterness, a pair of 2 hydrophobic groups or of hydrophobic and basic groups (Tamura *et al,* 1990). The B' site has to be open to produce bit-

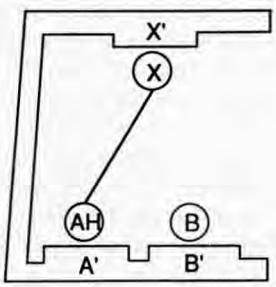
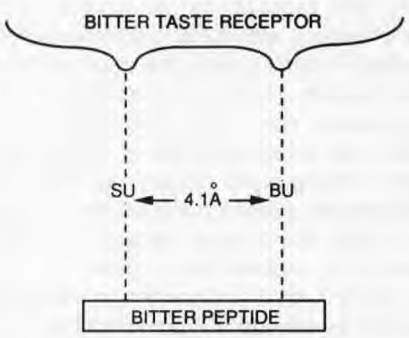


Fig 11. Bitter taste receptor model proposed by Shinoda and Okai (1985).
Modèle proposé par Shinoda et Okai (1985) pour un récepteur du goût amer.



Where BU: binding unit (hydrophobic group)
SU: stimulating unit (hydrophobic or basic group)

Fig 12. Scheme of binding of bitter peptide with bitter taste receptor (source: Ishibashi *et al,* 1988c).
Schéma représentant la liaison entre le peptide amer et le récepteur du goût amer (source: Ishibashi et al, *1988c).*

terness. AH-X components can thus be regarded as being bitter substances.

Supporting the preceding hypothesis, Ishibashi *et al* (1988c) have postulated a mechanism for bitter taste sensibility in peptides. The intensity of bitterness is thus assumed to be dependent on the features of the binding and stimulating units (the primary and the secondary bitter taste determinant sites, respectively) and on the distance between the units (AH–X) estimated to be 4.1 Å (fig 12). Small changes in this distance would probably affect the intensity of bitterness.

Bitterness of amino acids

Produced as a result of enzymic protein hydrolysis, the bitter taste may occur as an off-flavour during cheese maturation, and should therefore be considered undesirable. Some authors (Belitz *et al,* 1979; Gatfield, 1981) have established a relationship between the structure of the amino acids and bitter taste: 1) Configuration at the α-C-atom is important for the amino acid taste quality; while the natural L-forms of the hydrophobic amino acids are bitter to humans, the D-enantiomorphs are sweet. For both types of taste the presence of a hydrophobic area is essential, and the difference in taste sensation between the L- and D-enantiomorphs is due to the different spatial arrangement of $-NH_2$ and –COOH relative to the hydrophobic side chain. 2) The length of the side chain R is important both for the quality and intensity of taste (Ishibashi *et al,* 1988a): up to R = Et, D- and L-amino acids are sweet, while R > Et causes bitter taste of increasing intensity in the L-series and increasing sweet taste in the D-series. 3) Amino acids with branched side chains are significantly more bitter than the corresponding compounds with unbranched chains of equal C-number. 4) The presence of an ammonium group on the amino acid makes it able to be alkylated, arylated or acylated. Thus N-acetylation or esterification have been shown to abolish the sweet taste and to increase the bitter taste. However, the carboxylate group in an amino acid is not essential for bitter taste since its substitution with hydrogen or its esterification or amidation does not decrease the bitter taste. 5) Aromatic side chains generally increase bitter taste intensity. L-phenylalanine and L-tryptophan, for instance, are among the most bitter amino acids; in fact tryptophan is the most hydrophobic of all common amino acids and is approximately half as bitter as caffeine (Solms *et al,* 1965). According to Okai (1976) the hydrophobic side group of these aromatic amino acids may act as one of the bitter taste binding sites (fig 11). 6) The type and position of substitution of polar components in the side chain can also influence bitterness. Bitter taste is usually decreased by addi-

tion of amino, hydroxy, carboxylic or carboxamide groups, and the greater the distance between the α-amino group and the polar component, the smaller the contribution to bitter taste. 7) Apart from steric factors, the side chain hydrophobicity and hence the total amino acid hydrophobicity plays the most significant role in creating a perception of bitter taste (Ishibashi *et al,* 1988a). Threshold values of bitter amino acids leucine, tryptophan, phenylalanine, isoleucine, arginine, valine, methionine and histidine are listed in table VIII (Kirimura *et al,* 1969; Fujimaki *et al,* 1970a; Matoba *et al,* 1970).

Table VIII. Threshold values of bitter amino acids (from Kirimura *et al,* 1969).
Valeurs seuil des acides aminés amers (source : Kirimura et al, *1969).*

Amino acids	R_{caf}[1]	TV[2] *mmol.l⁻¹*		*Bitterness*[3] *(%)*
His	–	–	0.02	**
Arg.HCl	–	–	0.03	***
Met	–	–	0.03	***
Val	0.05	19	0.04	***
Arg	0.04	25	0.05	***
Ile	0.04	25	0.09	***
Phe	0.05	19	0.09	***
Trp	–	–	0.09	***
Leu	0.05	20	0.19	***
Pro	0.08	13	0.30	***

[1] The ratio of amino acid bitterness to caffeine, R_{caf}, was obtained by dividing the threshold value *(TV)* of caffeine (1.0) by those of amino acids (*eg,* Pro has a bitter taste equivalent to 0.08 that of caffeine) (Tamura *et al,* 1990); [2] *TV* = threshold value (mmol.l⁻¹ and %); [3] asterisks denote relative taste intensities. The taste intensities of individual amino acids were estimated by comparing them to that of solutions of NaCl at concentrations of 0.2 to 5.123 g per 100 ml.

[1] *Le quotient d'amertume des acides aminés par rapport à la caféine* R_{caf} *est obtenu en divisant la valeur seuil* (TV) *de la caféine (1,0) par celles des acides aminés (Pro a un goût amer équivalent à 0,08 fois celui de la caféine) (Tamura* et al, *1990);* [2] TV = *valeur seuil (mmol.l⁻¹ et %);* [3] *les astérisques indiquent l'intensité relative du goût. Les intensités des goûts des acides aminés sont estimées par comparaison avec celle d'une solution de NaCl à des concentrations de 0,2–5,123 g /100 ml.*

Bitterness in peptides

Although hydrophobicity (including the hydrophobic side chain, the kind and number of hydrophobic groups and the *Q*-value) has been previously shown to be the main factor involved in the determination of peptide bitterness, other factors such as the sequence and spatial structure are also suspected to play a role. However, these last 2 factors have not yet been fully studied.

With the exception of aspartic acid dipeptide esters (Wieser and Belitz, 1976), the bitter taste of peptides does not seem to depend on the configuration at the α-C-atoms of the amino acids involved, but on the amino acid sequence. However the literature on this subject is somewhat controversial (Arai *et al,* 1970a; Matoba *et al,* 1970; Matoba and Hata, 1972; Sullivan and Jago, 1972; Shiraishi *et al,* 1973; Gardner, 1978; Shinoda *et al,* 1985b). An essential requirement for bitter taste is the presence of at least one hydrophobic side chain (Matoba and Hata, 1972; Wieser and Belitz, 1976; Belitz *et al,* 1979; Ishibashi *et al,* 1987b).

Bitterness has been found in peptides ranging in size from 2 to 23 residues. It has been observed that dipeptides containing neutral amino acids with either large alkyl groups ($\geq C_3$) or a combination of large and small ($\leq C_2$) alkyl groups, neutral and aromatic amino acids, or neutral and basic amino acids, are bitter (Kirimura *et al,* 1969). One cause of bitterness lies in peptides, containing a high proportion of hydrophobic side-chains (proline, leucine and valine), as well as of glutamic acid, with a ratio of aliphatic to acidic amino acids of 0.8–1.3 (Edwards and Kosikowski, 1983), but these do not fully explain all the bitterness (Manning and Nursten, 1985).

As shown by their solubility in organic solvents, lipid solubility or hydrophobicity is the common property of all the bitter pep-

tides so far isolated. In contrast to sweet-tasting compounds, for which 2 polar groups complemented by a hydrophobic group are essential (bipolar-hydrophobic concept), bitter compounds require only 1 polar group, together with a hydrophobic moiety (monopolar-hydrophobic concept) (Belitz and Wieser, 1985).

It was earlier suggested by Ney (1971) that hydrophobicity was the only factor determining whether a peptide would be bitter or not; as mentioned above, it is possible to qualitatively predict the degree of peptide bitterness with the aid of Ney's *Q*-value, which, based on Tanford's studies, represents the average hydrophobicity of the amino acid side chains (Belitz *et al,* 1979; Wieser and Belitz, 1976; Otagiri *et al,* 1985). However, the accuracy of the estimation of bitter taste thresholds is limited because steric parameters, although important for the intensity of bitter taste, are not reflected in the total peptide hydrophobicity. Bitter peptides with an average hydrophobicity of less than 1 300 have thus been isolated (Kirimura *et al,* 1969; Fujimaki *et al,* 1970a).

The nature of the terminal amino acids in bitter peptides has been shown to have some significance. At least terminal leucine, isoleucine, phenylalanine, lysine and valine appear important for peptide bitterness (Kirimura *et al,* 1969; Sullivan and Jago, 1972; Schalinatus and Behnke, 1975b). A hydrophobic terminal residue in peptides functions as a bitter taste determinant site, and its location at the C-terminus has been reported by Okai (1976) to make the peptide more bitter than when at the N-terminus. Hydrophobicity from leucine, phenylalanine or tyrosine residues causes marked peptide bitterness. Intensity of bitterness is greater when leucine and phenylalanine are located at the C-terminus than at the N-terminus or in the middle of peptides (Ishibashi *et al,* 1988b), and also when their content is increased in peptides. Similarly, L-tyrosine gives rise to a bitter taste in peptides. However, the effect of tyrosine location is not as definite as in the case of phenylalanine peptides, probably due to the presence of a hydroxyl group in the tyrosine molecule (Ishibashi *et al,* 1987a, b). The bitter taste of peptides linking the C-terminal valine has been shown to be appreciably less than for those linking the N-terminal valine (Ishibashi *et al,* 1988a).

The contribution of L-proline residues to bitterness has been recognised from the study of synthetic di- and tripeptides. To give bitter taste to a dipeptide, a proline residue needs its partner amino acid to contain more than 5 carbons, and by means of the combination with other amino acids proline promotes bitter taste intensity (Shiraishi *et al,* 1973; Shinoda *et al,* 1986a); thus the most significant role of a proline residue in peptide bitterness does not depend on its hydrophobic character. Instead, conformational alteration of the peptide molecule by folding of the peptide backbone due to the imino ring of the proline residue ensures that the peptide molecule will form a suitable conformation for the bitter taste receptor. When the proline residue is located at the N-terminus the bitter taste is weak or absent. To produce bitter taste in proline peptides, 2 determinant sites (binding unit and stimulating unit) are essential and they should be adjacent in the steric conformation of peptides (Ishibashi *et al,* 1988b). A high content of proline dispersed along the peptide chain renders the peptide resistant to normal endo- and exopeptidase attack (Sullivan *et al,* 1973); bitter peptides have thus been found to be more resistant to enzymic degradation (Schalinatus and Behnke, 1975b). An arginine residue contiguous to proline, such as in Arg-Pro, Gly-Arg-Pro and Arg-Pro-Gly has resulted in a strong bitter taste, and in-

creasing the number of amino acids in the peptides whose structure is $(Arg)_x$- $(Pro)_y$ $(Phe)_z$ (where x = 1-2; y and z = 1-3) resulted in a synergistic effect for bitter taste (Otagiri *et al,* 1985).

Linking of 1 or 2 amino acids (glycine, alanine, valine, leucine) to the α- or ω-carboxyl groups of aspartic or glutamic acid has proved that although aspartic acid and glutamic acid are less perceptibly bitter (table VIII), they are able to elicit a bitter taste when they compose the peptides (Ohyama *et al,* 1988). Being a tasteless amino acid residue in peptides, glycine is limited to the role of spacer (Ishibashi *et al,* 1987a, 1988b). It is suggested, in order to strengthen peptide bitterness, that highly hydrophobic amino acids (tryptophan, isoleucine, tyrosine, phenylalanine; see table I) concentrate in the C-terminal, and basic amino acids (arginine, lysine, histidine) in the N-terminal of the molecule. Moreover, the presence of the valine residue, although increasing bulkiness and hydrophobicity, is less effective than that of basic amino acids. The limiting threshold value for bitterness of peptides composed of only neutral and hydrophobic amino acids seems to be 0.1 $mmol.l^{-1}$ (Shinoda *et al,* 1985a, 1986a, b). Cyclopeptides are more bitter than the corresponding open-chain peptides and taste intensity increases with hydrophobicity (Belitz and Wieser, 1985). Studies on various kinds of model bitter peptides have led Otagiri *et al* (1985) to report that bitterness potency generally increases with increasing molecular weight.

Size, amino acid composition and N-terminal residues of medium length peptides (4–10 residues) isolated from bitter Cheddar, Edam and Gouda cheeses at 4 months of age satisfy the requirements for bitterness (Sullivan and Jago, 1972).

The sensation of bitterness is caused by chemical compounds with a hydrophobic region and one hydrophilic group spaced 0.3 nm apart. This configuration is obeyed, among others, by the hydrophobic natural L-amino acids and peptides therefrom (Belitz *et al,* 1979). Peptide bitterness decreases upon complete hydrolysis to free amino acids. Since in hydrophobic peptides the amino and carboxyl groups are blocked and peptide bonds formed, they are considerably more bitter than the corresponding mixture of free amino acids; thus a peptide molecule with a high content of amino acids with hydrophobic side chains will also develop a bitter taste (Matoba and Hata, 1972; Adler-Nissen, 1984). Bitter peptides have been found to possess a higher content of aromatic (phenylalanine and tyrosine), aromatic-heterocyclic (tryptophan) and also heterocyclic (proline and histidine) amino acids (Roland *et al,* 1978). A protein such as casein is not bitter in itself, however, hydrolysis to peptides of varying size results in exposure of hydrophobic side chains which can interact with taste buds, increasing the bitter flavour. Bitterness is comparatively weak when a hydrophobic amino acid is in a terminal position, and weakest when it is free. Thus, an extensive hydrolysis will usually result in decreased overall bitterness (Adler-Nissen, 1986a). Out of 3 major fractions of casein, α_{s1}-, β-, and κ-, only α_{s1}-casein is degraded to any extent at the pH, NaCl and calcium lactate levels found in Cheddar cheese (Richardson and Creamer, 1970; Sullivan and Jago, 1972; Pélissier and Ribadeau-Dumas, 1976). β-Casein is more resistant to enzymic degradation because of its high content in proline residues (Phelan *et al,* 1973; Visser and de Groot-Mostert, 1977), and peptides derived from β-casein are therefore likely to be present at much lower levels. Since the C-terminal sequence of β-casein has been shown to possess an extremely bitter taste (Shinoda *et al,* 1985a), a higher content of hydrophobic amino acids in β-casein would thus make it a greater poten-

tial source of bitter peptides than α_s-casein. The β-casein component in a mixed system consisting of casein, rennet (or chymosin) and proteolytic enzymes from *Lactococcus lactis* subsp *cremoris* HP-starter bacteria has been shown to be the main source of bitter peptides (Visser *et al,* 1983a, b). It was earlier proposed that bitter peptides originated from β-casein (Sullivan and Jago, 1972); however, contrasting with the generally accepted belief that β-casein is the major source of bitter peptides in dairy products, it was found that cheese made from milk containing the *A* variant of α_{s1}-casein is less bitter because the N-terminal region of this protein is not degraded in the same way as in variants *B* and *C*; this would then suggest that bitter peptides present in normal Cheddar cheese largely originate from α_{s1}-caseins *B* or *C* (Richardson and Creamer, 1973) and not from β-casein. Knowing that α_{s1}-casein is almost nonexistent in goat milk and that products from total goat casein are always much less bitter than those from total cow casein, Pélissier and Ribadeau-Dumas (1976) have suggested α_{s1}-casein to be the main source of bitter peptides.

A bitterness map has been constructed from the β-casein sequence by Shinoda *et al* (1986a) for its potential use in food industry. These authors also suggest from the analysis of bitter peptides: β: 61–69; β: 46–47; β: 53–79, that some of the good bitter cheese components are derived from the middle portion of β-casein. In addition, it is noteworthy that these 3 bitter peptides isolated from cheese included a common hydrophobic fragment, β: 61–67: H-Pro-Phe-Pro-Gly-Pro-Ile-Pro-OH. A structure in which the hydrophobic side chains are directed towards the interior of the particle with one or more hydrophilic groups pointing towards the solvent has been suggested for β-casein bitter peptide fragments 193–209 and 193–207 (and/or 193–208), which combine high average hydrophobicity with good water solubility (Visser *et al,* 1983b).

Since it effectively blocks normal aminopeptidase breakdown, the presence of the N-terminal pyrrolidonecarboxylyl residue has been found to make bitter peptides accumulate in dairy products (Sullivan *et al,* 1973). Peptides containing a cyclised glutamate end-group have been shown to be bitter while removal of it made them lose their bitterness (Sullivan and Jago 1970; Fox and Walley, 1971). On the contrary, Hamilton *et al* (1974) observed that high salt concentrations in Cheddar cheese preserved the peptide β: 46–67 in its most bitter form, because N-terminal glutamine could not be easily converted to the pyrrolidone carboxylic acid (PCA) form.

Infrared spectroscopy has shown the structure of 2 kinds of bitter peptide prepared from enzymically and nonenzymically hydrolysed casein to be similar (Tokita, 1969a). This unique structure was formed partly by cyclic amide, γ-lactam or other lactam structures consisting of NH and CO groups produced by dehydration and hydrolysis during proteolysis.

Phosphopeptides and bitter peptides are both known to be resistant to proteolytic attack, while purified phosphopeptides from rennet casein hydrolysates or from Cheddar cheese extracts do not possess bitterness. However, since peptides prepared from the same sources were solely bitter when completely free of bound phosphate then phosphopeptides should not be considered responsible for the bitterness in Cheddar cheese (Dulley and Kitchen, 1972).

Structural information from studies on bitterness (determined organoleptically by a panel evaluation) of synthesised peptides (BPI-a, retro BPI-a, BPI-c, several analogs and a bitter peptide consisting of tryptophan and leucine) is given below.

H-Arg-Gly-Pro-Pro-Phe-Ile-Val-OH (BPI-a)

The characteristic conformation of peptide BPI-a, with a sequence slightly different from that of the C-terminal tail of β-casein (202-209), is responsible for its strong bitter taste, which requires the presence of at least 6 amino acid residues and one L-proline residue at the 3- position; however, the number of hydrophobic amino acid residues at the C-terminal is not important (Otagiri *et al,* 1983). On the other hand, results from Otagiri *et al* (1984) suggest both basic amino acid residues in N-terminal position and hydrophobic amino acid residues in the C-terminal moiety to be required for the intense bitterness of BPI-a; although Minamiura *et al* (1972b) showed earlier that this peptide is still bitter even upon removal of the C-terminal valine and isoleucine residues, and the N-terminal arginine. The strong bitterness exhibited by BPI-a was also found to depend on both basicity and side chain length of the N-terminal amino acid (Kanehisa and Okai, 1984). Synthesis of retro-BPI-a (possessing a reverse sequence of BPI-a) and its fragments has led Shigenaga *et al* (1984) to find that, in order for the bitter taste to be exhibited by BPI-a, the spatial structure of the molecule is more important than the position of the basic and hydrophobic moieties. In addition to hydrophobicity, spatial structure may thus be another factor in increasing the bitterness potency of BPI-a (Otagiri *et al,* 1985). Studies of Fukui *et al* (1983) and Otagiri *et al* (1983) on this peptide have also led them to conclude that specific amino acids as well as overall conformation of BPI-a is important to the intensity of bitterness. In addition, they suggest that at least 6 amino acids are necessary, and that glycine and L-proline are important for bitterness in this peptide.

It has been suggested by Aoyagi and Izumiya (1977) and Fukui *et al* (1983) to convert the sequence of the heptapeptide BPI-a to the correct C-terminal sequence of β-casein, the octapeptide β: 202-209. However, studies of synthetic peptides have helped to prove that the structure of the peptide BPI-a does not need to be converted to Ribadeau-Dumas *et al* (1972) formula: H-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH; this could be explained by another kind of variant in β-casein (Kanehisa *et al,* 1984).

H-Val-Tyr-Pro-Phe-Pro-Pro-Gly-OH (BPI-c)

It has been found that proline residues in positions 5 and 6 are necessary for the strong bitterness of BPI-c. The basic nature of the C-terminal and the hydrophobic nature of its N-terminal also contribute to the bitter taste of this peptide. In addition to the individual amino acids, some specific molecular configuration, *ie* tertiary structure, is also required for the strongly bitter taste of BPI-c, which has a bitter taste 20 times stronger than that of caffeine *ie* R_{caf} = 20 (Kanehisa, 1984).

A bitter peptide consisting of tryptophan and leucine

A bitter peptide isolated from casein digests (Minamiura *et al,* 1972b), with the putative chemical structure cyclo (L-leucyl-L-tryptophyl-L-Leucyl-L-tryptophyl) (Minamiura *et al,* 1972a), has also been synthesised for structural study. Results have shown that this peptide is a diketopiperazine, and its structure has been proved to be cyclo (L-leucyl-L-tryptophyl) (Shida and Nunami, 1974).

DISAPPEARANCE OF BITTERNESS IN PROTEOLYTIC PRODUCTS

Although bitterness has to be accepted as a necessary consequence of proteolysis it can nevertheless be mitigated by masking, removal, or prevention.

Masking or reduction of bitterness

Generally, enzymic protein hydrolysates have a bitter taste which may be one of the main factors limiting the spread of their utilisation in food. However, a low level of bitterness can, to a certain degree, be accepted as a necessary consequence of proteolysis because it may be masked by the use of different products: 1) bitterness has been masked through the use of glutamic acid and glutamyl rich oligopeptides (Desmazeaud and Hermier (1972); 2) addition of polyphosphates has successfully masked the bitterness of casein hydrolysates (Tokita, 1969b); 3) gelatin can also achieve a similar effect, although not as well as glycine (Stanley, 1981; Kilara, 1985); 4) Gly-Gly residues added to the C-terminal or N-terminal positions of bitter peptides (Shinoda *et al,* 1987); 5) cyclodextrin, because of its capacity for wrapping the hydrophobic functions of bitter amino acids. However, a large excess of α-cyclodextrin solution (150 $g.l^{-1}$) is necessary to substantially mask the bitterness of synthesised peptides (Tamura *et al,* 1990). Addition of 10% β-cyclodextrin is also effective in masking bitterness of skim milk hydrolysates (Helbig *et al,* 1980). However, further experiments are needed to establish its safety as a food additive. Until 1981, Japan was the only country where the use of cyclodextrin in food products was not limited (Szejtli, 1981). Monomeric and polymeric β-cyclodextrin are commercially available; however, among polymers from α-, β- and γ-cyclodextrins, β- seems to be the best in studies involving debittering of grapefruit juices (Shaw and Wilson, 1985; Shaw and Buslig, 1986); 6) starch: this sugar polymer is expected to cover the bitter compounds with "its net structure" and to prevent them from reaching the bitter taste receptor sites. However, for this purpose it is necessary to heat the mixtures of starch and bitter peptides at 100 °C overnight (Tamura *et al,* 1990); 7) proteins and peptides. Due to their affinity, addition of peptide compounds such as skim milk, soybean, casein, whey protein concentrate and casein hydrolysate to bitter amino acids and peptides is effective in decreasing or masking their bitterness (Tamura *et al,* 1990); 8) acidic amino acids. Bitterness can be masked by aspartic acid or glutamic acid but, on the negative side, they produce sourness (Noguchi *et al,* 1975). However, an acidic solution of taurine reduces amino acid bitterness as effectively as other acidic amino acids and that, without sourness. Bitterness of peptides is masked only when a large excess of amino acids is added (Tamura *et al,* 1990).

Casein digests obtained with the ficin/pepsin system have a very bitter flavour. Addition of pig's kidney homogenate as a source of exopeptidases (which remove amino acids singly from the ends of the peptide molecules) to the enzyme system raises the degree of hydrolysis, and yields casein digests relatively free of bitterness, containing small peptides and over 50% free amino acids (Clegg, 1973). Clegg and Mc Millan (1974) thus concluded that within the spectrum of the proteolytic enzymes in pig's kidney there is a system capable of hydrolysing the peptide responsible for the bitter flavour. It was even possible to modify this last method for the production of kilogram quantities of the relatively non-bitter casein hydrolysate for use in medical trials (Clegg *et al,* 1974b). Although Japanese

workers have been more concerned about bitterness in soybean than in milk products, it is possible to extrapolate their results to milk proteins. Thus bitterness appearing during an enzymic proteolysis was found to be caused by bitter peptides bearing hydrophobic amino acid residues (especially leucine) in the C-terminal structures, and to be lessened by decomposing these C-terminal structures with exopeptidases such as bovine pancreas carboxypeptidase A, *Aspergillus* acid carboxypeptidase and leucine aminopeptidase (Fujimaki *et al,* 1968; Yamashita *et al,* 1969; Arai *et al,* 1970a, b). However, according to Fujimaki *et al* (1970b), debittering methods using these exopeptidases encounter certain limitation, since such enzymes produce significant amounts of free amino acids, mainly hydrophobic, that may affect the food quality of the proteolysates (*eg* if the exopeptidases are not effective in removing the odours (Arai *et al,* 1970b)).

Bitterness due to peptides has also been shown to be reduced by applying the plastein reaction (Fujimaki *et al,* 1970c). Incubation, under appropriate conditions, of a partially hydrolysed protein such as casein with a certain proteolytic enzyme gives rise to the formation of a "plastein", a high-molecular protein-like substance whose properties are quite different from those of the original protein (Nakai and Li-Chan, 1988). This plastein reaction has been shown by Fujimaki *et al* (1970b) to be very effective in obtaining non-bitter, bland proteinaceous food materials. Being highly plastein-productive enzymes (nearly equivalent to α-chymotrypsin) Bioprase and Prozyme would be effective in debittering protein hydrolysates.

A reduction in the average hydrophobicity of peptides from casein digests treated with wheat carboxypeptidase has been observed by Umetsu *et al* (1983) and by Umetsu and Ichishima (1988). By releasing hydrophobic amino acids with a Δf value > 1 600 cal.mol^{-1} from the carboxyl termini of bitter peptides, wheat carboxypeptidase might thus be used to eliminate bitter taste in hydrolysates.

Research has been conducted by Minagawa *et al* (1988) into the specificity of a thermostable and metal-dependent aminopeptidase T from *Thermus aquaticus* YT-1. They found this enzyme to possess a broad substrate specificity, and to satisfactorily hydrolyse dipeptides and oligopeptides containing such hydrophobic amino acids as leucine, phenylalanine, and tyrosine at their N-terminals. It also hydrolyses peptides with N-terminal proline, but not dipeptides or tripeptides of the type containing a proline residue at a position second from the N-terminal. Later on, the same team of scientists (Minagawa *et al,* 1989) proved that aminopeptidase T was able to decrease and/or remove the bitterness of the peptide fractions present in casein hydrolysates obtained with 3 proteases (subtilisin, papain and trypsin). They could then assume that the bitterness formed during cheese ripening might be decreased by the action of aminopeptidase T, which would then contribute to shortening the ripening period of cheese by accelerating the hydrolysis of its constituent proteins and also to improving the cheese taste flavour.

Many attempts have been made to reduce bitterness in cheese manufacture. However, they have to be suited to the cheese type. Thus: 1) incubation of surface moulded soft cheeses (Camembert type) between the 3rd and 10th day following cheese-making in a slightly ammoniacal atmosphere has led to a faster increase of the pH in the outer layer. The growth of *Penicillium caseicolum* was then less rapid, proteolysis less intense and bitterness decreased (Vassal and Gripon, 1984); 2) bitterness of semi-liquid slurries of Cheddar cheese curd induced by a neutral microbial proteinase from *Bacillus subtilis* has disappeared with the addition of

an intracellular peptidase extract from *Lactococcus lactis* subsp *lactis* (NCDO 712; "Accelase") (Cliffe and Law, 1990); 3) addition of heat-treated culture of *Lactobacillus helveticus* enhanced the peptidolytic activity of a Swiss round-eyed semi-hard low-fat cheese and thus reduced bitterness (Ardö *et al,* 1989). The same effect has been observed by Bartels *et al* (1987a) for a Gouda cheese to which viable cells of *Lactobacillus helveticus* CNRZ 32 were added; 4) the lactic acid bacterium *Lactococcus lactis* subsp *cremoris* is known to possess a debittering activity. A study conducted by Piët *et al* (1990) has led them to conclude that the composition of the fermentation medium influences neither the debittering activity of this bacterial strain nor the level of proteolytic activity of the cell-free extracts.

Removal of bitterness

Techniques for the removal of bitterness include adsorption on food grade resins or activated carbon, chromatographic procedures (Helbig *et al,* 1980), solvent extraction (*eg,* butanol; Matoba *et al,* 1970) or bioconversion to compounds which may be nonbitter or of diminished bitterness. However, removal is the choice of last resort after dilution or masking because of the added expense. Moreover, it is considerably more difficult to remove bitter compounds from solid foods because most procedures involve some form of liquid-solid extraction. Therefore, because of economic constraints or toxicological considerations, commercial methods for removal of bitter peptides are not available (Kilara, 1985).

Modification or arrangement of structure

By blocking the amino group of bitter amino acids such as leucine, valine and phenylalanine, and peptides by the introduction of acetyl, aspartyl and glutamyl residues, Tamura *et al* (1990) have proved that the simplest way to remove the bitterness is to change the structure of these compounds. Incubation of protein hydrolysates with a neutral bacterial protease in the presence of added non-bitter amino acids such as alanine, glycine, serine and glutamic and aspartic acids makes these amino acids result, *via* transamination, in peptides of reduced bitterness (Rouseff, 1990).

Chromatographic procedures

Supporting the hypothesis that hydrophobic compounds are mainly responsible for the bitter taste of protein hydrolysates, hydrophobic interaction chromatography (HIC) on hexylsepharose gel can be used for debittering casein digests (Lalasidis and Sjöberg, 1978). However, the limited capacity of hexylsepharose gel to bind the bitter compounds indicates a need to develop other hydrophobic gel types with improved capacity and properties.

Hydrophobic affinity chromatography with butylepoxy and alkylamino Sepharose gels is less effective in debittering casein hydrolysates than when hexylepoxy and octylepoxy Sepharose gels are used (Helbig *et al,* 1980). Sephadex G-10 and G-25 gels, and hydrophobic adsorbents such as Sephadex LH-20 and phenoxyacetyl cellulose are also effective in respectively reducing some bitterness from casein hydrolysates, and up to 50% from skim milk hydrolysates (Helbig *et al,* 1980). Roland *et al* (1978) have developed a hydrophobic chromatography process for debittering casein hydrolysates. For this purpose they used a phenol-formaldehyde resin polymer (Duolite S-761) which interacts preferentially with the nonpolar side chains of the aromatic and heterocyclic amino acids present in the peptides. Although having

some limitations concerning the high proportion of talc relative to casein the low flow rate and temperature conditions, adsorption chromatography on talc (Chersi and Zito, 1976) has also been suggested for debittering casein hydrolysates (Kilara, 1985).

Solvent extraction

Some methods listed in the literature involve extraction of enzymic hydrolysates with different solvents (azeotropic secondary butyl alcohol, aqueous ethanol and aqueous isopropanol) to remove or reduce their bitter taste.

Azeotropic secondary butyl alcohol (SBA)

When a protein hydrolysate is extracted with SBA the result is a selective transfer of hydrophobic peptides and amino acids to the SBA-phase, while more hydrophilic peptides and amino acids remain in the aqueous phase. Since peptides containing these hydrophobic amino acids have a bitter taste, it is reasonable to assume that the reduction of bitterness is mainly or wholly due to removal of these compounds. Indeed, the material recovered from the SBA-phase, following the extraction of casein and whey hydrolysates, has been found to have an exceptionally strong bitter taste (Lalasidis, 1978).

Aqueous ethanol (AE) and aqueous isopropanol (AI)

Extraction of protein hydrolysates with different concentrations of ethanol or isopropanol has been effective in removing bitter compounds. Results have shown the amount of protein hydrolysates dissolved in the AE- or AI-phase to increase with the concentration of water in the mixture, while there was a gradual reduction of the content of essential amino acids in the alcohol insoluble fraction. The bitter material was concentrated in the AE- or AI-phase, which had a strong bitter taste (Lalasidis, 1978). Removal of bitter peptides from enzymic fish protein hydrolysates has also been done with AE by Chakrabarti (1983). In addition, he suggested using this method to remove bitter fractions from concentrated filtrates of protein hydrolysates (*eg* casein) prior to vacuum drying, thus eliminating the cost of initial vacuum drying, and also minimising handling cost.

Other methods

Glass materials such as soft glass fiber, flint glass powder, and microfiber paper have been shown to have some adsorption capacity for bitter peptides from skim milk hydrolysates, while adsorption is negligible on plastic such as low density polyethylene and polyvinylidene chloride (Helbig *et al,* 1980).

Activated carbon treatment, previously shown to reduce the bitterness of a commercial casein hydrolysate (Murray and Baker, 1952), has also been suggested as a promising technique for improving the palatability of casein and skim milk hydrolysates (Helbig *et al,* 1980). Digests produced in the latter study were found suitable for use in beverage fortification. Addition of activated carbon at a level of 0.5 g/g of protein hydrolysate made Cogan *et al* (1981) successful in debittering casein hydrolysate. However, they also noticed a loss of 26 ± 2% of protein nitrogen, which was mostly due to the selective adsorption by the activated carbon of tryptophan and phenylalanine, or peptides containing them. Since this last treatment is commonly used in protein hydrolysate manufacture as a means of decolorisation, it could also accomplish debittering (Olsen and Adler-Nissen, 1979).

Avoidance or prevention of bitterness

Since the proteolytic cleavage of milk proteins often results in the formation of bitter tasting peptides after extensive hydrolysis, control and termination of the hydrolytic reaction at a desirable degree of hydrolysis is crucial to avoid or prevent the formation of bitter peptides (Vegarud and Langsrud, 1989).

Bitterness can be prevented by chemical modification of functional side-chains in amino acids of proteases such as subtilisins, chymotrypsin or trypsin. Thus, nitration, iodination, glutarylation, succinylation or modification of carboxyl groups lead to changes in the enzymic properties with regard to the hydrolysis of polypeptide substrates. However, the concerted effort of kinetic, crystallographic, and chemical studies is still necessary for a more complete picture of the interactions of proteolytic enzymes with their natural substrates, the proteins (*eg* caseins), to obtain more valuable information both about the size and the specificity of the active sites. Proteases compatible with the substrates could thus be used. Choosing alcalase, a protease known to cleave proteins at the carboxylic side of hydrophobic amino acids, would result in less bitterness than if the cleavage were carried out by a protease cleaving at hydrophilic amino acids (Svendsen, 1976). Thus, according to Pélissier and Manchon (1976), Adler-Nissen (1986c) has elaborated on the influence of the protease used for the production of enzymic hydrolysates on the development of bitterness. He thus concluded that theoretically hydrophobic proteases (*eg* alcalase) will produce peptides with an average hydrophobicity which will decrease with an increment of the peptide chain length. Non-hydrophobic proteases (*eg* trypsin), on the contrary, will produce peptides with a relative excess of hydrophobic amino acids in endopositions and with an average hydrophobicity increasing with peptide chain length. In reality, however, predictions about the bitterness of peptides cannot be made with regard to the effect of protease specificity. This arises especially for the long peptides, which may form loops and clusters and thereby reduce their bitterness, and also if a separation is carried out at the isoelectric point.

CONCLUSION

Bitterness, which can be a problem in just about all foods –fruits, vegetables, protein products, processed and fermented products– is reasonably well correlated with the degree of molecule hydrophobicity. The sensation of bitterness requires the simultaneous participation of a number of different papillae. In order for the compound to produce a response detectable as bitterness, the conformation of this compound must be sufficiently specific and have sufficient binding energy to distort the microstructural conformation of the receptor membrane.

This review article has been mainly concerned with this sensory defect in cheese (formation, isolation, identification, structure, masking or inhibition). Based on the different hypotheses found in literature and on the work carried out in our laboratory, we propose a new model for the development of bitter peptides in Cheddar cheese (fig 13). This model is mainly concerned with the development of the Cheddar flavour and the elimination of bitter peptides in Cheddar cheese. In a first step, long chain non-bitter peptides are obtained from the action of rennet and non-starter *Lactobacillus*. Starter enzymes are presumed to further attack these long chain non-bitter peptides and release bitter polypeptides. Addition of debittering lactic bacteria dur-

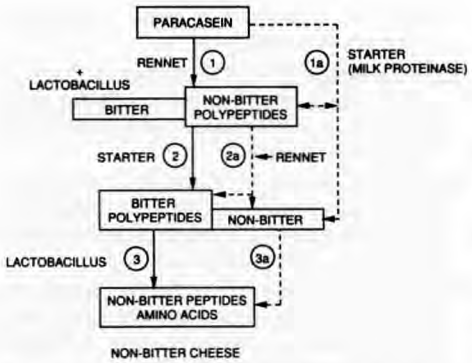


Fig 13. Proposed model for the production of non-bitter Cheddar cheese.
Modèle proposé pour la fabrication de fromage Cheddar non-amer.

ing cheese manufacture has an important effect. Indeed, being rich in aminopeptidases and dipeptidases, dipeptidyl peptidase and carboxipeptidase (only for *Lactobacillus casei*), these bacteria, especially *Lactobacillus*, are then able to hydrolyse bitter peptides and produce a Cheddar cheese devoid of bitter off-flavour. More studies will be necessary to determine whether it is appropriate for Cheddar cheese manufactured with pasteurised milk and under conditions of accelerated ripening.

As new analytical techniques and equipment are developed, information about the identity of the bitter principle and the mechanism of its formation is being accumulated and will help to clarify many unresolved issues of importance to the scientist. Thus, recent techniques of high-performance liquid chromatography coupled to fast atom bombardment-mass spectrometry will allow isolation, molecular weight and sequence determination of peptides from milk products (Lemieux and Amiot, 1991). Important properties for making peptides extremely bitter are high hydrophobicity, basic amino acids in the amino end and presence of proline; moreover, intermediate-sized peptides could be expected to be more bitter than the smaller one. Although procedures developed to remove or reduce bitterness in food products such as soy can be applied or adapted to milk products, many of them are still not practicable or economical for commercial use, or involve compounds which have not been approved for food use. The most effective methods for bitterness control in cheese involve reduction of total proteolytic activity by culture selection, use of mixed cultures, proper selection and use of coagulant, and adjusting environmental factors (*ie* temperature, pH, etc). Thus it is now possible to manufacture non-bitter cheese with accelerated ripening and produce dietetic enzymic casein hydrolysates with reduced bitterness.

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