Flavour peptides: the potential role of Lactococcal peptidases in their production

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Lactic acid bacteria, cheese making and biotechnology

For thousands of years man has used the natural bacterial flora of milk to prepare dairy products such as cheese and yoghurt. Starter cultures, now used by the dairy industry for these products, developed by using the milk inoculum that gave good-flavoured cheeses and transferring it on in subsequent cheese making. Many different methods of cheese making have now been developed with over 400 varieties, although there are only about 18 distinctly different types. There are three main classes of cheese, soft, blue-veined and hard-pressed, although all originate from a lactic fermentation with milk. This acidification of the milk lactose to lactic acid is one of the major functions of the starter culture and hence the bacteria involved are called lactic acid bacteria (LAB). The major genera of LAB used in the dairy industry are Lactococcus (formerly Streptococcus), Lactobacillus, and Leuconostoc, with the former, in particularly Lactococcus lactis subspecies cremoris and lactis, being the prevalent type used for hard cheese manufacture [1].

As these LAB have been used for many generations they are generally regarded as safe (GRAS) organisms and it is argued that the enzymes from these sources will be acceptable to the food industry for biotechnological processes. There is therefore significant interest in these organisms and their enzymes with the view to their exploitation in new areas of food biotechnology.

Existence of flavour peptides

From other food sources, polar hydrophilic peptides have been reported to have flavour properties. Glutamyl oligopeptides such as Glu-Asp, Glu-Glu, Glu-Ser, and Glu-Gly-Ser, isolated from a proteinase-modified soy bean protein, were the first peptides demonstrated to have favourable taste properties [2]. Similar acidic oligopeptides were found when fish protein concentrate was hydro-
lysed with Pronase producing an acidic fraction of molecular mass less than 1000 Da (but not free aspartate and glutamate) that was reported to be brothy with favourable after taste [3]. Further analysis of this fraction revealed over 30 oligopeptides from dipeptides to hexapeptides with a high glutamate and other hydrophilic amino acid content [4].

A peptide with the sequence, Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala, has been isolated from the gravy of beef meat that is reported to have a delicious flavour [5]. Known as 'delicious' peptide, its flavour properties have been confirmed by chemical synthesis [6]. Studies on the taste active sites revealed the peptide had an umami and a sour taste and that the overall taste was caused by an interaction between the basic and the acidic components [7]. This work also showed that di- and tripeptide fragments of the delicious peptide when combined in the correct quantities had the same taste threshold as the parent molecule. This result indicates that interactions between small peptides produced in protein hydrolysates may be a significant factor to be considered in future studies.

**Cheese and flavours**

Some cheeses, such as cottage cheese, are consumed fresh and rely on the starter culture strain, *Lactococcus lactis* subsp. *lactis* (formerly known as *Streptococcus diacetylactis*), to develop flavour during the fermentative process by breaking down diacetyl, a byproduct of pyruvate. Hard cheeses such as Cheddar and Gouda, however, rely on a ripening stage where changes within the cheese lead to the complex formation of sensory components. These ripening processes are thought to be brought about by enzymatic breakdown of the milk components: lactose, lipids and proteins, mainly the caseins. The major source of these enzymes for ripening are the LAB, but also important are the indigenous enzymes in milk (plasmin) and the gel-forming enzyme, chymosin from rennet, initially used to form the curd.

The chemical components involved in the development of flavours during ripening of hard cheeses such as Cheddar, despite numerous publications on cheese flavour and aroma, have yet to be properly elucidated. Although the peptide-containing water soluble fraction of cheese is acknowledged to make the greatest contribution to the intensity of cheese flavour [8], progress is just beginning to occur on the flavour properties of peptides in cheese. The significance of this research is likely to be the development of new, naturally derived, flavour compounds which may be of greater consumer acceptance.

**Peptides in cheese flavour**

The best characterized peptides involved in flavour of cheese are those associated with bitterness [9]. Bitter peptides have now been isolated and characterized from cheeses such as Cheddar [10, 11] and Gouda [12] and also from protein hydrolysates formed by the action of chymosin [13] and trypsin [14] on caseins. The hydrophobic amino acid content of the peptides is the major factor contributing to their bitterness. From synthetic studies peptides with a high hydrophobic content were demonstrated to be more bitter than their constituent free amino acids [15]. The actual sequence of the hydrophobic amino acids in the bitter peptides was considered not crucial although blocking of the N-terminal amino and C-terminal carboxyl groups increased bitterness. In cheeses, bitterness is considered to be a defect and starter culture strains are now developed to limit this characteristic.

For desirable flavours there is little direct evidence for peptides being the flavour components. A cyclic dipeptide of asparagine and phenylalanine has been patented that is the active ingredient in a composition intended to give the aroma and mouth-feel of cheese [16]. Mostly, however, the evidence for flavour peptides in cheese is inferred from the accelerated cheese ripening work where the use of proteinase combined with a peptidase-containing LAB extract increased the cheese maturation rate. One such system, proposed by Law and Wigmore in 1983, uses a neutral proteinase from *Bacillus subtilis* and a *Streptococcus lactis* NCDO 712 intracellular extract to accelerate the ripening process [17]. This achieved a balanced increase in the rate of formation of savoury flavour notes in Cheddar cheese compared with controls. The peptidic nature of the flavour components is inferred by the necessity for the initial protease reaction to successfully accelerate the ripening.

The structure of these flavour components in Cheddar cheese and the enzymes involved in their generation is still unknown. The proposed mechanism for accelerated ripening is based on findings that neutral proteinase supplements the initial proteinase activity acting on the caseins, but causes the production of undesirable bitter cheeses when used in high concentrations [18, 19]. This is presumed to be owing to the production of hydrophobic bitter peptides. The addition of the intracellular extract of *S. lactis* NCDO 712 was proposed as a method, using the peptidases therein, of breaking down these
peptides. This combination of proteinase and peptidases leads to the development of the normal Cheddar cheese flavour in a significantly faster time than in control cheeses.

**Flavour peptides in cheese; fractionation of the water-soluble fraction**

To further investigate the role of peptides in cheese flavours we have recently developed a method for preparing and chromatographically analysing the peptide-containing water-soluble fraction of cheeses [20], which makes the greatest contribution to the intensity of cheese flavour. This system involved the preparation of a water/cheese slurry from which, following centrifugation, the supernatant is partially deproteinated by methanol precipitation and defatted with a hexane extraction. The aqueous extract is then concentrated and applied to a Sephadex G-25 column. A typical chromatogram is shown in Fig. 1. The peptide- and amino acid-containing fractions were then either separated into bands, as shown in Fig. 1, or pooled as one, before analysis by reverse phase h.p.l.c.

This system was then used to study the effects of proteinases and peptidase-containing extracts on the flavouring of cheese curds slurries [21]. Figure 2(a) shows a reverse phase chromatogram of an untreated cheese curd slurry, producing few peaks in the bland-tasting material, which are probably

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**Fig. 1**
Sephadex G-25 gel permeation chromatography of the water-soluble nitrogen fraction from Cheddar cheese

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**Fig. 2**
Reverse phase h.p.l.c. analysis of the water-soluble nitrogen fraction of cheese curd slurries following incubation with accelerated-ripening enzymes

(a) The cheese curd slurry before addition of enzymes. (b) The cheese curd slurry following incubation with Neutrase 1.5S (1.5 Anson units/g) for 7.5 h. (c) The curd slurry from (b) further treated with S. lactis NCDO 712 extract (4.7 units/g) for 21 h.
the result of limited proteolysis of the curd by the chymosin. When the slurry was treated with Neutrase, a proteinase with a broader bond specificity than chymosin, the resulting chromatogram (Fig. 2b) showed many peaks, many very late running, indicating hydrophobicity and therefore bitterness. Tasting of the slurry confirmed this bitterness. This bitterness disappeared when the slurry was further treated with a peptidase-containing extract from \textit{S. lactis} NCDO 712, giving a normal Cheddar flavour to the curd slurry. In Fig. 2(c) the resulting chromatogram of the slurry shows the decrease in the later-running hydrophobic components found in Fig. 2(b) and an increase in the earlier-running, more hydrophilic components. These incubations took place in hours rather than the months required for ripening Cheddar cheese and further supports the concept that protein hydrolysis, mainly of the milk caseins by the proteolytic enzyme system of the starter culture, plays a crucial role in the generation of desirable flavours within the cheese.

To identify the actual flavour peptides of the cheese, we had to adjust the conditions for preparing the water-soluble extract to allow tasting. This involved deleting the hexane extraction and performing an ethanol precipitation rather than methanol. The resulting Sephadex G-25 chromatogram (Fig. 1) was essentially the same as previously found and the fractions were pooled according to the elution absorbance profile. Following concentration by rotary evaporation, each fraction was tasted. As anticipated the higher-molecular-mass fractions (bands 2 and 3) had a distinct bitter taste, thought to be caused by the hydrophobic peptides which have previously been isolated from cheeses [10-12]. The later running fractions (bands 4, 5 and 6) which corresponded to the elution range for tripeptides down to amino acids had distinct savoury/brothy flavours. This agrees with the cheese slurry work where the development of a more savoury flavour correlated with the disappearance of the bitter hydrophobic peptides and the appearance of the further hydrolysed and therefore smaller hydrophilic peptides.

Reverse phase h.p.l.c. analysis of these bands confirmed this, with the bitter fractions, bands 2 and 3, having a substantial amount of later running hydrophobic material. These bands are very complex with over 30 recognized peaks on the chromatogram. Bands 4, 5 and 6, with desired flavours have more hydrophilic components present, although they are still complex, with between 10 and 30 peaks on the chromatogram; some are very early running on reverse phase h.p.l.c. and are probably mixtures. We are now trying to isolate the individual components from the chromatograms to identify those with flavour properties, although practical problems exist regarding the tasting of materials isolated using the solvents associated with chromatography.

\textbf{The proteolytic system of LAB}

LAB are nutritionally fastidious and require amino acids and peptides for growth. These are often sub-optimal in media such as milk, and LAB have developed a complex proteolytic system comprising proteases, peptidases and transport systems to provide these nutrients from high protein media, enabling rapid growth. Some of the enzymes of the LAB proteolytic system have been isolated and characterized. Table 1 shows the peptidases from \textit{Lactococci} that have been isolated and characterized to date. This is generally considered to be incomplete with some peptidase and proteinase activities having been described in crude and partially purified extracts, but not yet attributed to an isolated enzyme [37], thus preventing understanding of the complete process of total protein hydrolysis.

The peptidase activities found in LAB may also be dependent on the conditions used during growth of the culture. The use of a high protein milk medium, leads to the expression of the cell envelope proteinase activity to break down the milk proteins and thus supply the LAB with essential nutrients. This expression can be suppressed when a high-peptide-containing medium is used [38]. Whether or not other enzymes are controlled by similar mechanisms remains unknown but such studies will play an important role in any attempts to genetically manipulate LAB with respect to proteinases or peptidase expression.

As it is not yet known which enzymes from LAB are responsible for the formation of the flavour products, a knowledge-based approach to this field is required where the hydrolysis can be shown to be brought about by a particular set of defined enzymes. This can come from two directions. The first is to isolate and characterize the actual flavour components and determine which enzymes are likely to lead to their formation. The second is to isolate sufficient amounts of the proteinases and peptidases from LAB and determine their actions on the food proteins, both individually and in sequential actions on a substrate, analysing by organoleptic and chemical methods.
Table I
Peptidases isolated from Lactococci

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specificity</th>
<th>Nature of residue X</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino peptidase(s)</td>
<td>x~000</td>
<td>Broad range</td>
<td>[22-24]</td>
</tr>
<tr>
<td>Amino peptidase A</td>
<td>x~000</td>
<td>Asp, Glu</td>
<td>[25, 26]</td>
</tr>
<tr>
<td>Dipeptidase</td>
<td>o~o</td>
<td>Dipeptides</td>
<td>[27-29]</td>
</tr>
<tr>
<td>Tripeptidase</td>
<td>x~00</td>
<td>Tripeptides</td>
<td>[30]</td>
</tr>
<tr>
<td>X-Pro dipeptidyl peptidase</td>
<td>ox~00</td>
<td>Pro at 2nd residue</td>
<td>[31–33]</td>
</tr>
<tr>
<td>Prolidase</td>
<td>o~x</td>
<td>X-Pro dipeptide</td>
<td>[34]</td>
</tr>
<tr>
<td>Endopeptidase (LEP-I)</td>
<td>oo~xo</td>
<td>N.D.</td>
<td>[35]</td>
</tr>
<tr>
<td>Endopeptidase (LEP-II)</td>
<td>oo~xo</td>
<td>Hydrophobic</td>
<td>[36]</td>
</tr>
</tbody>
</table>

Debittering enzymes
As desirable flavour peptides from cheese have yet to be confirmed, a second possibility exists, that the LAB proteolytic enzymes may also act by debittering, breaking down the hydrophobic bitter peptides that may be masking the more desirable flavours. This in itself may be a significant role for these enzymes both in cheese making and in future proteolytically-derived flavour hydrolysates. Analysis of the proteolytic activities of starter strains known to develop both bitter and good cheeses is underway. Profiling of these activities may well show which enzymes need to be present in abundance to assist removal of the bitter peptides or form desirable flavour peptides.

Micro-organisms as a novel source of flavour compounds
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Consumer demand for natural flavour ingredients for foods and beverages has resulted in substantial research in production of these ingredients via processes which are considered to be natural. A number of research papers and patents describing these approaches have been published [1–3]. In this paper, the use of micro-organisms for commercial production of natural flavour compounds is discussed.

Appropriate micro-organisms may be obtained from the many culture collections based on information available in the literature or can be isolated from nature using selective screening techniques. A survey of disclosed commercial processes reveals that in most cases a natural precursor was used which is closely related structurally to the product. De novo biosynthesis from substrates such as carbohydrates, acetate, etc., in most cases has not resulted in viable commercial processes. In other words, it seems that controlled catabolic transformation of a precursor is preferred over de novo biosynthetic pathways for production of natural flavour compounds.

The historical development of industrial biotechnology may be divided into four eras [4]: (1) production of foods, such as wine, beer, vinegar, cheese, yogurt, bread, etc.; (2) large-scale production of specific materials under non-sterile conditions (e.g., organic acids, solvents, biomass, etc.), by relatively simple technology; (3) production under sterile conditions, of relatively expensive secondary metabolites by more complex processes (e.g., penicillin); and (4) application of modern scientific developments in biotechnology, e.g., enzyme research, gene technology, molecular biology and process engineering. Enzyme research has led to processes involving immobilized enzymes and whole cells. Application of developments in the field of molecular biology and gene technology have led to processes using micro-organisms with specifically altered gene structure. Process engineering has led to the development of new reactors, optimization of processes, and better measurement and control of process parameters. In the near future bioengineering advances may lead to computer control of entire production processes from inoculation to product recovery.

There are several requisites for industrial micro-organisms [5]: purity, genetic stability, amenability to change by mutagenic agents, facile formation of reproductive units, acceptable growth rate, and relatively short process time for product accumulation. In addition, if possible, the strain should protect itself against contamination, be capable of long-term culture maintenance, and ideally, minimize or eliminate production of all toxic substances.

Such micro-organisms may either be obtained from a culture collection or isolated from natural sources by screening programmes. Screening may

References


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