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The Biotechnological Development of New Food Preservatives

S. ROLLER

Biotechnology Unit, Leatherhead Food Research Association, Leatherhead, Surrey, UK

Introduction

The food-poisoning micro-organisms of major concern today are those that contaminate foods and cause illness by infection (*Listeria monocytogenes, Yersinia enterocolitica, Vibrio parahaemolyticus*, numerous *Salmonella* species, enteropathogenic strains of *Escherichia coli, Campylobacter jejuni* and *coli* and *Clostridium perfringens*) and by forming toxins (*Staphylococcus aureus, Clostridium botulinum, Bacillus cereus*, and some strains of *Bacillus subtilis* and *licheniformis*). Reported cases of salmonellosis and listeriosis increased steadily during the 1970s and 1980s, with an especially sharp rise in some countries during the past 5 years associated with outbreaks in certain foods (Baird-Parker, 1990; Jones, 1990). In addition, there is a plethora of spoilage organisms, too numerous to list here, that cause wastage and losses of food during processing, transport and storage. Thus, the use of added preservatives as one of the many options available for extending the shelf-life of foods is likely to remain an important activity in food product formulation. Indeed, while consumer pressure is forcing many food companies to reconsider and reduce the levels of usage of certain food 'additives', the need for effective food preservatives is greater than ever and is fuelling a renewed interest in microbial inhibitors. It is in this search for hitherto unknown, more effective antimicrobials, tailor-made to combat specific spoilage or pathogenic organisms, that the role of biotechnological techniques will be most evident in the future.

In the following review, the impact of recent advances in traditional biotechnology (fermentation and enzymology), as well as in the newer sciences of molecular biology (genetic manipulation and protein engineering) on the development of new food preservatives will be examined.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; UV, ultraviolet.

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Preservatives produced by fermentation

ORGANIC ACIDS

Organic acids such as acetic, lactic, citric, fumaric, malic, tartaric and gluconic acids are used extensively in foods such as soft drinks, alcoholic beverages, salad dressings, baked products, jams, jellies, ice-cream, processed cheese and confectionery. These edible acidulants are indispensable to the food industry not only for their preserving action but also for the characteristic flavour they give to foods. However, most organic acids are produced by traditional fermentation techniques, some of which have not changed for decades. Although it is conceivable that improvements in strain stability and product yields could be made using recombinant DNA technology, the relatively low cost of food acidulants makes it unlikely that biotechnological techniques will have a major impact on this business sector. Therefore, further discussion of the organic acids as food preservatives will not be undertaken here. The interested reader is referred to reviews by Buchta (1983a, 1983b), Ebner and Follmann (1983), Rohr, Kubicek and Kominek (1983a, 1983b), Ruttloff (1987) and Dziezak (1990).

MICROBIAL PEPTIDES

Small, usually cationic, peptides with pronounced antimicrobial activity are common in nature and many have been described from animal, plant and microbial sources (Sahl, 1985). In spite of their different sources, these peptides display structural similarities including molecular masses of between 3000 and 6000 and isoelectric points of approximately 10. Peptides that are easily degraded in the human digestive tract hold an important potential safety advantage over chemically synthesized preservatives that may enter the bloodstream. On the other hand, sensitivity to proteolytic enzymes can constitute a disadvantage if the peptide is to be used in foods such as fresh meat. Furthermore, many peptides would not be suitable for use as food preservatives, owing to the pathogenic nature of some of the microbial producers. For example, the best understood group of antimicrobial peptides, the colicins, are produced by *E. coli*, an organism usually associated with poor hygiene in the food industry. Further examples are the peptides epidermin and Pep 5, produced by strains of *Staph. epidermidis* (Ersfeld-Dressen, Sahl and Brandis, 1984; Horner *et al.*, 1989), which may be useful as antimicrobial agents in topical applications, such as in creams and salves, but are unlikely to receive regulatory approval for use in foods without extensive (and expensive) toxicological evidence that the products are safe to ingest. Nevertheless, several peptides with antimicrobial spectra useful in foods have been identified; some have been commercialized and many more show good potential for future commercialization.

NISIN

Nisin is produced by *Lactococcus* (formerly *Streptococcus*) *lactis* and belongs to a class of compounds known as the bacteriocins. These have been defined by Tagg, Dajani and Wannamaker (1976) as proteinaceous compounds with a bactericidal action against a limited range of organisms usually closely related to the producer organism. In addition, bacteriocin production is usually, but not always, governed by plasmid-borne genetic determinants. For recent reviews on the biology, chemistry, toxicity and food applications of nisin, the reader is referred to papers by Delves-Broughton (1990a, 1990b).

Although discovered as long ago as 1928, nisin was not used in foods until 1951 when Hirsch *et al.* demonstrated the ability of nisin-producing cultures to prevent cheese spoilage by clostridial gas formation. Indeed, the ability of nisin to inhibit growth of Gram-positive bacteria such as *Staphylococcus* and spore outgrowth of several species of *Clostridium* and *Bacillus* has been its most useful property. In addition, recent work by Benkerroum and Sandine (1988) suggests that it may be possible to use nisin in the control of the important food pathogen, *L. monocytogenes*. Furthermore, the combination of nisin with the chelating agent EDTA and/or the surfactants Triton X-100 and Tween 20 has been shown to be effective against a number of Gram-negative bacteria, including *Salmonella typhimurium* and *E. coli* in laboratory media and a model food system based on chicken (Blackburn *et al.*, 1989).

In 1969, nisin received clearance from the Joint FAP/WHO Expert Committee on Food Additives for use in foods and is now positively allowed by regulatory authorities in 47 countries. Nisin has been, and is, used as an effective preservative in processed cheese and cheese spreads, dairy desserts, canned food and, when refrigeration facilities are inadequate (as in some Middle Eastern countries), in pasteurized milk (Delves-Broughton, 1990a). Attempts have also been made to use nisin as an alternative preservative system to nitrites in cured meats, but the levels of addition necessary to inhibit *Cl. botulinum* have been too high to make the process economic (Calderon, Collins-Thompson and Usborne, 1985; Taylor and Somers, 1985; Taylor, Somers and Krueger, 1985; Bell and de Lacy, 1987). Today, nisin remains the only bacteriocin produced by lactic acid bacteria that is commercially available.

Nisin has been shown to contain 34 amino acid residues including the unusual lanthionine, and its molecular weight has been determined as 3510 with some evidence of dimer (MW 7000) and tetramer (MW 14 000) formation in solution (Gross and Morell, 1971). The antimicrobial activity of nisin is optimal under acidic conditions and is reduced by heat treatment and storage in foods. It has been suggested that the mode of action of nisin is based on the disruption of the cytoplasmic membrane (Morris, Walsh and Hansen, 1984).

The possibility that nisin production may be plasmid-mediated was suggested by several investigators in the 1970s to early 1980s. For example, Le Blanc, Crow and Lee (1980) reported phenotypic and physical evidence for a 28 MDa nisin plasmid in *L. lactis*. In 1984, Gasson linked nisin production

and resistance to a 30 MDa plasmid and successfully transferred the trait to a non-producing, plasmid-free strain of *L. lactis*. Further studies have shown that the final nisin structure was subject to post-translational modification of a precursor polypeptide by enzymic modification of cysteine, threonine and serine residues (Dodd, Horn and Gasson, 1990). The gene sequences of nisin and its structural analogue subtilin (from *B. subtilis*) have recently been elucidated by scientists at the University of Maryland (Banerjee and Hansen, 1988; Buchman, Banerjee and Hansen, 1988; Hansen, Banerjee and Buchman, 1989). These studies provide the background data essential for the development of a range of useful bacteriocin analogues tailored against the spoilage and pathogenic flora of specific foods. For further details of the genetic work on nisin and other bacteriocins, the interested reader is referred to papers by Larry McKay, Todd Klaenhammer and Mike Gasson (for example, Gasson, 1984; Gasson and Anderson, 1985; Klaenhammer, 1988; McKay, 1989).

BACTERIOCINS OTHER THAN NISIN

Examination of a sufficiently large number of strains (that is, 100 or more) of any one species of micro-organism is generally rewarded with some evidence of antagonism. A quick glance through the contents pages of any microbiological journal published in the past few years will reveal numerous papers reporting the existence of bacteriocins, particularly from lactic acid bacteria. However, this group of organisms also produces a host of other metabolites with inhibitory properties and these can be the source of many errors in the screening procedure. For example, the production of hydrogen peroxide by lactic acid bacteria is well documented (Whittenbury, 1964; Anders, Hogg and Jago, 1970; Premi and Bottazzi, 1972; Collins and Aramaki, 1980) as is its inhibitory effect on *Staph. aureus* (Dahiya and Speck, 1968; Attaie *et al.*, 1987), pseudomonads (Price and Lee, 1969) and salmonellae (Mulder, van der Hulst and Bolder, 1987). In addition, many screening assays are based on active cells, which produce an abundance of lactic and acetic acids; these primary metabolites are the main substances responsible for the antimicrobial properties of lactic acid bacteria. Although many authors have used these primary metabolites as controls individually, the effect of a combination of primary metabolites has often been ignored. Correct controls for each individual strain must be constructed following analysis of the fermentation broth for all the primary metabolites. Using a very carefully controlled screening procedure, ten Brink, Bol and Huis in't Veld (1988) found that only a handful of strains out of 1000 screened produced bacteriocins. However, the lessons of the past are slow to be learned and systematic studies of the benefits and pitfalls of different assay methods are only just beginning to appear in the scientific literature (Spelhaug and Harlander, 1989).

For a detailed examination of the literature on the biochemistry and genetics of some of the better-characterized bacteriocins reported up to mid-1987, the review by Klaenhammer (1988) is recommended. The bacteriocins reviewed by Klaenhammer included lactocin 27 and helveticin J from

strains of *Lactobacillus helveticus*, lactacin B and F from *Lactobacillus acidophilus*, plantaracin A from *Lactobacillus plantarum*, diplococcin and Las 5 from *Streptococcus cremoris*, the lactostrepcins from *Streptococcus lactis*, and pediocin A from *Pediococcus pentosaceus*. Other authors have also reported the production of acidophilin from *Lactobacillus acidophilus* (Shahani, Vakil and Chandan, 1972; Shahani, Vakil and Kilara, 1976, 1977) and bulgarican from *Lactobacillus bulgaricus* (Reddy and Shahani, 1971; Reddy *et al.*, 1983). However, as acidophilin had not been characterized as proteinaceous, it is uncertain whether this compound could be classified as a bacteriocin by Tagg's 1976 definition. Numerous additional bacteriocins have been described since the Klaenhammer review, including, for example, sakacin A and lactocin S from strains of *Lactobacillus sake* (Schillinger and Lucke, 1989; Mortvedt and Nes, 1990) and leucocin A from a strain of *Leuconostoc gelidum* isolated from refrigerated meat (Harding and Shaw, 1990; Hastings and Stiles, 1991).

Except for pediocin, all of the above bacteriocins have been shown to possess a very narrow inhibitory spectrum. The organisms producing these narrow-spectrum inhibitors may find uses in the construction of starter cultures for traditional food fermentations, in which they would compete more effectively with the lactic acid bacteria in the natural microflora and therefore produce fermented products with more consistent quality than is currently possible. However, scale-up, isolation and purification of these bacteriocins are unlikely to be undertaken by industry, owing to the limited number of foods in which the inhibition of lactic acid bacteria alone would be advantageous.

On the other hand, the broader-range bacteriocins such as pediocin A from *P. pentosaceus*, with reported activity against the food-borne pathogens *Staph. aureus*, *Cl. botulinum*, *Cl. perfringens*, *B. cereus* and *L. monocytogenes* and the food-spoilage agents *Streptococcus faecalis* and *Clostridium sporogenes*, hold more promise from the industrial point of view (Fleming, Etchells and Costilow, 1975; Daeschel and Klaenhammer, 1985; Daeschel, 1989). Another broad-spectrum pediocin, named pediocin AcH, from *Pediococcus acidilactici*, has been shown to be active against food-spoilage bacteria such as *Pseudomonas putida* and *Brochothrix thermosphacta* and pathogens such as *B. cereus*, *Cl. perfringens*, *L. monocytogenes* and *Staph. aureus* (Bhunia, Johnson and Ray, 1988). Furthermore, partially purified pediocin AcH has been shown to be non-immunogenic to eight mice and a rabbit (Bhunia *et al.*, 1990). Mode-of-action studies suggest that pediocin AcH acted on sensitive Gram-positive target cells by binding to specific receptors on the cell envelope, followed by disruption of the integrity of the cell membrane with consequent cell death (Bhunia *et al.*, 1991).

Although the development of the pediocins as food preservatives is currently hindered by low levels of production, difficulties in purification and genetic instability (Klaenhammer, 1988; Barnby-Smith *et al.*, 1989; Barnby-Smith and Roller, 1990), some recent publications suggest that it may not be long before these problems are resolved. Thus, it has been claimed that a preparation of pediocin PA-1 obtained from *P. acidilactici* by centrifugation

and filtration of the culture, followed by ammonium sulphate precipitation and dialysis, was effective in preventing the spoilage of a salad dressing inoculated with 10^3 CFU g^{-1} of *Lactococcus fermentans* and stored at room temperature for 7 days (Gonzales, 1989). The dressings were assessed by smell and taste and were found to be satisfactory. The inoculated control dressing spoiled after 5 days' storage. Using the same producer organism as Gonzales (1989), Nielsen, Dickson and Crouse (1990) tested the efficacy of a crude preparation of pediocin (the microbial culture was centrifuged, neutralized and filtered) on beef surfaces contaminated with *L. monocytogenes*. The bacteriocin was shown to have a bactericidal mode of action, reducing the number of *L. monocytogenes* by 0·5–2·5 log cycles, depending on bacteriocin concentration, numbers of listeria present and the time of application of the bacteriocin (before or after contamination). There appeared to be no inactivation of the bacteriocin by the proteases normally present in fresh meat; however, this may be explained by the low temperature (5°C) of storage. Further work in other food applications is awaited with interest.

An alternative approach to extracting and purifying the antagonistic compounds has been to add whole, bacteriocin-producing cultures or centrifuged, 'cell-free' culture preparations to foods. However, this approach has met with limited success (Gibbs, 1987; Pucci *et al.*, 1988; Gombas, 1989; Berry *et al.*, 1990; S. Roller, unpublished). For example, Berry and co-workers (1990) have found only marginal differences in the survival of *L. monocytogenes* in fermented semi-dry sausage supplemented with 10^7 CFU g^{-1} of non-producing and bacteriocin-producing pediococci. On the other hand, it has been reported that the addition of several strains of *Lactobacillus* species at a level of 10^7 CFU ml^{-1} to model food systems inhibited the growth of moulds, psychrotrophic spoilage organisms and pathogens such as *Salmonella newport* and *L. monocytogenes* when stored at 7°C (Boudreaux, Matrozza and Leverone, 1989). As in studies with known food preservatives, it would appear that data obtained from inhibitory assays in laboratory media often cannot be extrapolated to food systems.

One of the disadvantages of adding whole cultures of lactic acid bacteria to foods for preservation purposes is that this group of organisms produces copious amounts of lactic and acetic acids. Whereas these primary metabolites may be desirable in fermented foods, where they play an important role in flavour formation, in fresh foods (e.g. fresh beefburgers) they have been shown to lead to undesirable organoleptic effects (Gibbs, 1987). Thus, it has been proposed that the antibacterial properties of lactic acid bacteria could be exploited in fresh foods by curing the organisms of their genetic determinants for organic acids. Gonzales, of Microlife Technics, has patented a method for using selected strains of *Strep. lactis* subsp. *diacetylactis* that have been cured of the ability to ferment lactose by removal of a 41 MDa plasmid (Gonzales, 1986). The patent claims that the modified organisms, added at a level of 10^5–10^6 CFU g^{-1}, were particularly active in cottage cheese against the spoilage organisms *Pseudomonas fragi* and *Pseudomonas fluorescens* inoculated at a level of 10^3 g. In addition, milk inoculated with the cured organisms

remained fresh after 12 days' storage at 10°C, whereas the control milk spoiled after 10 days (Gonzales, 1986).

The Wisconsin bacon process has been one of the few examples where the addition of a live culture of lactic acid bacteria to a non-fermented food has been a success. However, in this process, the preservative action was attributable to the production of acid by the pediococci. Thus, work carried out in the early 1980s at the University of Wisconsin showed that the level of sodium nitrite in bacon could be reduced from the recommended 120 p.p.m. to 80 and 40 p.p.m. without exposing consumers to an increased risk of botulism (Tanaka *et al.*, 1985a, 1985b). This was accomplished by incorporating *P. acidilactici* and a fermentable sugar (0.7% sucrose) into the curing mixture. When the bacon was temperature-abused, the lactic acid bacterium utilized the sugar to produce lactic acid, which lowered the pH and thus prevented the growth of *Cl. botulinum* (Anon., 1984a, 1984b). By 1986, ABC Research Laboratories were marketing 'Bacon Blend' for reducing nitrites (and thereby nitrosamines) in processed meats (Anon., 1986).

OTHER MICROBIAL PRODUCTS

Reuterin, a broad-spectrum antimicrobial substance produced by *Lactococcus reuteri* has recently been described (Talarico *et al.*, 1988; Talarico and Dobrogosz, 1989). Reuterin has been characterized as a highly soluble, pH-neutral mixture of monomeric and dimeric forms of β-hydroxypropionaldehyde produced during anaerobic fermentation of the organism on glycerol. The product was reported to be active against several species of *Salmonella, Shigella, Clostridium, Staphylococcus, Listeria, Candida* and *Trypanosoma*, and preliminary results suggest promising preservative activity in refrigerated ground beef (Daeschel, 1989).

ENGINEERED PEPTIDES

The immunoproteins of insects are a recently described group of peptides with antimicrobial activity which may have potential for exploitation as food preservatives in the future. In response to bacterial infection, the pupae of the *Cecropia* moth produce 10–15 immune proteins, including lysozyme, the cecropins A, B and D (MW around 4000) and the attacins (MW around 20 000). These compounds have been studied extensively by research groups in Stockholm and Uppsala, Sweden (Hultmark *et al.*, 1980, 1982, 1983; Boman, 1986). The cecropins have been shown to be active against both Gram-positive and Gram-negative bacteria, including pathogens of medical significance such as *Pseudomonas aeruginosa*. The attacins were active primarily against *E. coli* and their mode of action was based on the disruption of the outer membrane (Engstrom *et al.*, 1984). The Swedish group concluded that the three immune proteins cecropin, attacin and lysozyme appeared to act in concert, resulting in the effective inactivation of a whole range of structurally different micro-organisms.

Clearly, only minute quantities of the inhibitory peptides can be isolated

from the insect. However, scientists at Ingene Inc. in California have recently reported the successful cloning and expression of a gene encoding for a fusion protein incorporating cecropin A into *E. coli* (Lai *et al.*, 1989). It has been necessary to construct the gene encoding for a fusion protein rather than a simple cecropin peptide in order to protect the host organism from the product's antimicrobial properties once the gene is expressed. Following a fermentation cycle, the *E. coli* cells were harvested and homogenized, and the inclusion bodies containing the fusion protein were sedimented. The inclusions were solubilized and the cecropin released by acidification. Further purification by ion-exchange chromatography was required to produce food additive quality cecropin. Limited toxicity tests carried out by the company have shown no adverse effects of cecropin on sheep red blood cells, human B-cells and human fibroblast cell lines at 300 $\mu g\ ml^{-1}$ or on mice following feeding trials of up to 1·5 $g\ kg^{-1}$ body weight per day in a three-week period. The recombinant cecropin A, however, had a carboxyl group at the C-terminus where the insect-derived cecropin had an amide group; consequently, it was found that the recombinant cecropin also lost its antimicrobial activity against Gram-positive bacteria but not against Gram-negative bacteria. Ingene scientists have suggested chemical methods for attaching amide groups to the recombinant product to produce 'cecromycin' with antibacterial activity against a wide spectrum of Gram-positive and Gram-negative organisms. Thus, it would appear that the application of recombinant DNA technology may, in the future, allow the production of economically feasible quantities of the material.

Enzymes as preservatives

LYSOZYME

Although several forms of lysozyme are known to exist in nature, it is the hen albumen lysozyme that has been most studied and that is commercially available (Wilkins and Board, 1989). Lysozyme is a muramidase that cleaves the β-1,4-glycosidic bond between *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid in cell walls of Gram-positive bacteria. Recently, it has been demonstrated that lysozyme was effective against *L. monocytogenes* in laboratory media (Osa *et al.*, 1990). Used alone, avian lysozyme is inactive against Gram-negative bacteria but milk lysozyme has been reported to lyse *E. coli* and *P. aeruginosa* if the outer membrane is first removed by sodium chloride and EDTA, respectively (Vakil *et al.*, 1969). Thus, it is conceivable that the bactericidal spectrum of lysozyme could be extended by design, for example by using it as a preservative in food formulations that already contain agents, such as polyphosphates, known to interfere with the outer membrane of Gram-negative organisms. Similarly, sublethal heat treatments or freeze/thaw injury may predispose Gram-negative organisms to the bactericidal action of lysozyme (Wilkins and Board, 1989). Recent work on the shelf-lives of mayonnaise, confectionery cream filling and fruit mousse has demon-

strated synergistic effects between lysozyme and calcium sorbate, resulting in extension of shelf-lives of up to 50% (Lück *et al.*, 1988). In addition, there is some indication in the literature that lysozyme may attack chitin, an important constituent of the fungal cell wall (Tokura, 1989).

The commercially available lysozyme from hen egg white has a molecular weight of 14 400 and contains 129 amino acid residues with four intramolecular disulphide bonds (Scott, Hammer and Szalkucki, 1987). Hen egg white contains approximately 3·5% lysozyme, of which about 80% is readily extracted by ion-exchange chromatography.

The 'late blowing' of cheese (formation of unsightly holes in hard cheeses during maturation) by *Clostridium tyrobutyricum* is a recurrent spoilage problem in cheese-producing areas. In France alone, the cost of spoilage of Swiss-type cheeses has been estimated at around $US7 million per year (M. Maitenaz, Institut Technique du Gruyère, personal communication). Scott, Hammer and Szalkucki (1987) have estimated that in 1984 100 tonnes of lysozyme were used to prevent the late gas blowing of cheese in Europe. Although nitrate has traditionally been used to prevent late blowing, it is not permitted for use in cheese in the USA, and concern over the possible formation of carcinogenic nitrosamines has encouraged the use of alternative preservatives such as lysozyme. Lysozyme is usually added to milk destined for cheese production at a level of 25 mg l^{-1} to give a final concentration of 300 mg kg^{-1} cheese. The enzyme has been shown to arrest the growth of vegetative cells once germinated from spores (Carminati *et al.*, 1984). Interestingly, lysozyme has not been reported to interfere with the subsequent growth of lactic starter cultures except on a few occasions. Lysozyme has been shown to be particularly effective in Edam and in the Italian cheeses Provlone, Grana, Emmenthal, Asiago and Montasio (Wasserfall, Voss and Prokopek, 1976; Carini and Lodi, 1982).

There has been some interest recently in the possible application of lysozyme in infant formulae in order to achieve a similar balance of faecal flora to that found in breast-fed babies (Wharton, 1982; Wilkins and Board, 1989). However, results of trials have been inconclusive, probably because hen egg lysozyme, which is less active than milk lysozyme, was used (Reiter, 1984).

It has been suggested that the cost of lysozyme could be reduced if it were produced from a micro-organism by using fermentation. Bacteriophage T4 lysozyme has been a particularly attractive target as it has been shown to be 250 times more active against *E. coli* than the avian enzyme (Grutter and Matthews, 1982). Although structurally homologous to hen egg lysozyme, T4 lacks the intramolecular disulphide bonds (Hawkes, Grutter and Schellman, 1984). However, scientists at Genentech and Genencor have used protein engineering techniques to introduce a disulphide bond into the T4 lysozyme which increased its heat stability (Perry and Wetzel, 1984). More recently, the genes encoding for hen egg white lysozyme have been successfully transformed into *E. coli* and *Saccharomyces cerevisiae*, although yields (1–2 mg l^{-1}) of the recombinant protein have been disappointingly low (Kumagai *et al.*, 1987; Miki *et al.*, 1987). Higher levels (up to 12 mg l^{-1}) of

recombinant lysozyme secreted by *Aspergillus niger* have been reported by Archer *et al.* (1990), although this is still too low for a viable industrial process. Nevertheless, these problems do not appear insurmountable and rapid future developments can be anticipated.

Several native microbial *N*-acetylmuramidases similar to lysozyme have been subjected recently to trials in foods. Thus, Hayashi *et al.* (1989) have shown that 0·11% of a purified muramidase from *Streptomyces rutgersensis* extended the shelf-life of an adzuki bean paste (called '*an*' and used for making traditional Japanese sweets) from 48 to 109 h when stored at 10°C. The enzyme was shown to be effective against lactic acid bacteria, which constitute the predominant spoilage flora of *an*.

LACTOPEROXIDASE

Mammalian secretions such as milk and saliva contain low concentrations of peroxidases, which, coupled with thiocyanate ion and hydrogen peroxide, form hypothiocyanate, an unstable but highly reactive biocide. This rather elegant antimicrobial system has been exploited in a toothpaste that utilizes the endogenous peroxidase and thiocyanate in saliva with exogenous amyloglucosidase (to produce glucose) and glucose oxidase (to produce hydrogen peroxide from glucose and oxygen) (Hoogendoorn, 1974). It has also been recommended that the optimal activity of the endogenous lactoperoxidase in milk can be obtained by the addition of 12 and 8 p.p.m. of thiocyanate and hydrogen peroxide, respectively (Reiter and Harnulv, 1984). Indeed, the lactoperoxidase system has been used successfully in the preservation of raw milk in Sri Lanka, Pakistan, Kenya and Mexico when refrigeration prior to pasteurization was not available. The lactoperoxidase system was activated by the direct addition of 10 p.p.m. hydrogen peroxide to the raw milk (Banks, Board and Sparks, 1986). Hydrogen peroxide could also be generated *in situ* by glucose oxidase, although this option tends to be too expensive for application in developing countries. Nevertheless, it has been suggested that a combination of β-galactosidase and glucose oxidase immobilized on a column could be used as a means of 'cold sterilization' of milk (Bjorck *et al.*, 1975; Banks, Board and Sparks, 1986). However, at levels of peroxide addition used in some countries (300–500 p.p.m.), the lactoperoxidase system is destroyed and the biocidal action stems directly from the peroxide (Bjorck, Claesson and Schulthess, 1979).

Reiter (1984) has shown that the lactoperoxidase system is effective against the Gram-negative *E. coli*, *S. typhimurium* and *Ps. aeruginosa*. Recently, the lactoperoxidase system has also been shown to inhibit growth of *L. monocytogenes* in skimmed milk subjected to temperature abuse for 6 h at 30°C and 20 h at 20°C (Bibi and Bachmann, 1990). The only commercial sources of lactoperoxidase to date are raw milk and whey (Anon., 1985; Burling, 1989). The enzyme, however, together with co-produced milk proteins, has already shown some promising results in feeding trials with young calves: lactoperoxidase-fed animals appear to be less prone to diarrhoeal diseases than control animals, with consequent improvements in weight gain (Gud-

mundsson, 1984 (cited in Scott, Hammer and Szalkucki, 1987)). Supplementation of infant milk formulae with commercially available lactoperoxidase has also been shown to delay the onset of growth of *S. typhimurium* in the reconstituted product for 3 days and *E. coli* for 1 day at 15°C (Earnshaw *et al.*, 1990); although this represents a useful possible application of lactoperoxidase in countries where infant feed may be prepared with contaminated water, it is unlikely that the supplementation would currently be economic.

OTHER ENZYMES

Lytic enzymes with specific activity against fungi would be particularly attractive for development as food preservatives because many of the antimicrobials in current use are poor inhibitors of this class of organism. Although lysozyme has been shown to have some activity against fungal chitin (Tokura, 1989), its activity has not been sufficiently pronounced to warrant widespread application studies.

Chitin, a homopolymer of *N*-acetylglucosamine, is the major component of fungal cell walls. Therefore, it follows that an enzyme specific for fungal chitin may be useful as an inhibitor of common food-borne spoilage and pathogenic moulds. However, chitinases have not been tested against food-borne fungi. Enzymes of the chitinase complex have received substantial attention recently, mainly for the degradation of the naturally occurring and abundant polymer, chitin, and also because of their involvement in the resistance mechanisms of plants to pathogenic fungi. Thus, a number of chitinase/chitosanase enzymes have been described and, in some cases, isolated and characterized from higher plants (Oishi, Fumiyasu and Masao, 1989) and from *Streptococcus olivaceoviridis* (Diekman, Tschech and Plattner, 1989), *Bacillus circulans* (Yabuki, 1989), *Mucor rouxii* (Pedraza-Reyes and Lopez-Romero, 1989) and *Neurospora crassa* (McNab and Glover, 1989), to name but a few. Recently, stable chitinase-overproducing mutants of the fungus *Aphanocladium album* have been isolated following one-step UV mutagenesis (Vasseur *et al.*, 1990). Two chitinases, one from *Serratia marcescens* and another from *Streptococcus griseus* are also available commercially.

Another enzyme, glucose oxidase, which catalyses the oxidation of glucose to gluconate and hydrogen peroxide in the presence of molecular oxygen, has frequently been mistaken for a novel antimicrobial compound. Glucose oxidase is ubiquitous in nature and has been found in honey (White, Subers and Schepartz, 1963) and fungi (Kwang-ae Kim, Fravel and Papavizas, 1990). Combinations of glucose oxidase and catalase have also been found effective, with varying degrees of success, in reducing total viable counts and extending shelf-life when sprayed onto the surfaces of hamburger patties, sausages and smoked rainbow trout and packaged under modified atmospheres (Aaltonen, Lehtonen and Karilainen, 1990).

Enzymically prepared products as preservatives

SUCROSE ESTERS

The antimicrobial activity of fatty acids and their esterified derivatives has been studied extensively, and useful reviews of the earlier literature can be found in works by Kabara (1979, 1983). Most of these compounds are produced chemically and are used extensively by the food industry as emulsifiers. However, a small selection of these, including sucrose esters, are amenable to enzymic methods of synthesis and are therefore reviewed here.

The work of Klibanov at the Massachusetts Institute of Technology has been instrumental in changing our perception of what enzymes can and cannot do. Thus, in 1983, Zaks and Klibanov reported the successful reversal of the degradative action of lipase when used in a hydrocarbon solvent. Synthetic reactions carried out in food solvents (e.g. hexane), low-water systems or supercritical carbon dioxide hold much promise for the future of food ingredient technology. In addition to offering opportunities in the flavourings area (Gatfield, 1988), the reverse action of hydrolytic enzymes could be applied to the synthesis of novel sucrose and carbohydrate esters with antimicrobial as well as emulsifying properties. Although the latter has been a difficult target, recent reports suggest that new breakthroughs are imminent (Poole *et al.*, 1989; Godtfredsen, 1990; Janssen *et al.*, 1990).

Badel *et al.* (1988) have shown that sucrose esters of mono- and dipalmitate and stearate possess bacteriostatic properties against *Staphylococcus* spp., *Strep. faecalis*, *E. coli* and *Proteus morganii* in laboratory media incubated at 35°C. The minimum inhibitory concentrations ranged from 0·05 to 1·25 mg ml^{-1} and were lower in the presence of surfactants such as Tween 80 and Triton X-100. It is likely that the surfactants played a role not only in increasing the surface area available for the esters to exert their antimicrobial effect but also as potentiators of inhibition by their action on the cell envelope of the Gram-negative bacteria. However, control experiments containing the surfactants only were not carried out by the authors. Furthermore, Marshall and Bullerman (1986) have shown that mixtures of sucrose esters substituted with palmitic and stearic acids inhibited the growth of several mould species from *Aspergillus, Penicillium, Cladosporium* and *Alternaria* at an addition level of 1% in laboratory media. Aflatoxin production by *Aspergillus parasiticus*, however, was not inhibited by 0·1% of sucrose ester.

A recently marketed application of sucrose esters has been in the extension of shelf-life of fresh fruit and vegetables (Anon., 1990a). The new product, Semperfresh, has been prepared from a proprietary combination of sucrose esters, palm oil and cellulose to form an invisible, tasteless and odourless film. Semperfresh acts essentially as a barrier to oxygen and water, and somewhat less so to carbon dioxide, consequently extending the ripening time of the produce (Anon., 1990b). The shelf-life of pineapples stored at ambient temperatures has been extended from 11 to 28 days, while bananas remained fresh for 4 weeks instead of the more usual 2 weeks, following coating of the

fruit with Semperfresh. Water loss from refrigerated mange-tout peas has also been inhibited by the coating (Anon., 1990b).

Plant and algal products

It has been suggested that plant cell tissue culture could be used to produce valuable chemicals, including antimicrobial food ingredients (Evans and Whitaker, 1987). Although plant cell culture cannot, at present, compete with bacterial or yeast batch cell culture for cost of production, certain unique compounds, synthesized exclusively by plants, may become commercially important in the future. Furthermore, the potential for public acceptance of food ingredients prepared by plant cell culture is good in the light of an apparent consumer demand for more 'natural' foods or foods to which chemically synthesized preservatives have not been added.

Plants produce an impressive array of antimicrobial compounds, some of which have been recognized, if not necessarily characterized chemically, since ancient times. For detailed reviews of the area, the reader is referred to Hargreaves *et al.* (1975), Beuchat and Golden (1989) and Leadbetter (1991). The active ingredients responsible for the antimicrobial activity in herbs and spices have generally been found in the esssential oil fraction and have been identified as mixtures of esters, aldehydes, ketones, terpenes and phenolic compounds. Much recent interest has centred on the terpene class of compounds—notably eugenol from cloves and thymol from thyme. However, although shown to be active in laboratory trials (Jay and Rivers, 1984), the flavours associated with these materials have limited their use in foods. Similarly, some plant-derived pigments, such as the anthocyanins, have been shown to possess antimicrobial activity (Beuchat and Golden, 1989) but are unlikely to find application in foods where high pigmentation is undesirable. Nevertheless, it may be possible to enhance the antimicrobial potential of plant-derived flavouring and colouring compounds in foods in which the microbial flora has been subjected to additional stresses, such as, for example, heat. Thus, it has been shown that heat-stressed yeasts had increased sensitivity to the essential oils and crude solvent extracts of allspice, clove, garlic, onion, oregano, thyme and cinnamon (Conner and Beuchat, 1984, 1985).

In response to infection or injury, many plants are also known to produce phytoalexins, low molecular weight compounds with broad-spectrum antimicrobial activity (Dixon, 1986). Although many phytoalexins have been shown to be toxic to animal cells in tissue culture, it is conceivable that, once more is known about their structure/activity relationships, analogues without the undesirable toxic elements could be prepared (Wilkins and Board, 1990).

The use of plant cell culture for the production of antimicrobial compounds is clearly still in the future. Nevertheless, the first products of plant cell culture, such as shikonin, the purple pigment from *Lithospermum erythrorhizon*, are already on the market in Japan and more products are likely to follow.

ALGAE

Of the 150 000 species of marine algae estimated to exist, approximately 30 000 have been identified, although even the basic taxonomy of some of the commonly harvested species has not been completed (Harvey, 1988). The better-known macroalgae or seaweeds, the *Gelidium, Gracilaria, Chondrus* and *Macrocystis*, are still hand-collected from the world's oceans for their agar, carrageenan and alginate. The microalgae, a large and diverse group of photosynthetic micro-organisms, also comprise several thousand species (Borowitzka, 1988). Of these, fewer than 50 species have been studied in detail with respect to their physiology, biochemistry and potential for mass culture and industrial exploitation.

The microalgae, macroalgae and cyanobacteria (blue-green algae) produce a host of antibacterial substances, and the reader is referred to a paper by Jones (1986) for a more extended review of the subject. However, the exploitation of many algal antibiotics will be heavily dependent on the ability to mass cultivate these organisms.

Between 1974 and 1981, scientists at the Roche Research Institute of Marine Pharmacology (RRIMP) near Sydney, Australia, screened extracts from marine macroalgae for biologically active chemical substances with potential for application in the medical and veterinary fields. Initial screening was carried out on crude extracts obtained from frozen algae using a range of aqueous and non-aqueous solvents. The extracts were tested *in vitro* against several human pathogens (three Gram-positive bacteria, four Gram-negative bacteria, two yeasts, two fungi and one protozoan). Those extracts shown to have promising activity were also tested *in vivo* in laboratory mice. Of the 159 species of marine plants screened, 118 (74%) showed *in vitro* activity against one or more of the microbes used in the screen. However, of the crude extracts subsequently tested *in vivo*, only nine (5%) showed activity (Reichelt and Borowitzka, 1984).

In the Reichelt and Borowitzka study (1984), several interesting compounds active *in vitro* were isolated from the Rhodophyta (red algae) and found to be generally halogenated. However, a number of these were toxic to mice. By far the highest proportion of inhibitory activity was found in the genus Cystophora of the Phaeophyta class (brown algae) when tested against Gram-positive bacteria. Much of this activity was attributed to phenolic compounds that commonly occur in brown algae. Some of the compounds with *in vivo* activity were identified, e.g. an alkylated resorcinol from *Cystophora torulosa*, a phloroglucinol from *Cystophora scalaris* and a δ-tocotrienol from *Cystophora expansa*. Phenolics are generally considered to be toxic compounds; however, the presence of an alkyl group, such as in the algal phenolics, is known to render them less toxic. In addition, the Australian group observed that many of the algal antimicrobials identified in their study were rapidly eliminated in the mouse by either excretion or metabolism, a property that is undesirable in pharmaceuticals but may be highly beneficial in food applications. Studies on the *in vivo* activities of these

compounds were not completed, owing to the closure of the Institute by Roche (Reichelt and Borowitzka, 1984).

Mabrouk *et al.* (1985) have shown that five marine macroalgae of the Phaeophyta class (*Sargassum despiense, Turbinaria decurrense, Dilophus ligulatus, Cystoseira myrica* and *Padina pavonia*) inhibited mycelial growth and aflatoxin production by a toxigenic strain of *Aspergillus flavus*. The algae were prepared by washing fresh biomass with tap-water, drying at 40°C until constant weight was reached and finely grinding the product. Concentrations of algal powder ranging from 10 to 200 g l^{-1} of culture medium were tested in shake-flask culture. The volatile constituents of the Phaeophyta have been shown to contain fatty acids from C1 to C10, including acetic and propionic acids; lower terpenes, such as linalool and geraniol; and phenolic compounds such as *p*-cresol. The preservative activity of all of these compounds is well known. Other compounds that may also contribute to the preservative potential of algal extracts include fatty acids with longer chain lengths (C10–C20), sterols and triterpenes.

It has been claimed that both cell extracts and extracts of the spent growth media of the microalgae *Chlorella vulgaris* and *Chlamydomonas pyrenoidosa* have antimicrobial activity against Gram-positive and Gram-negative bacteria. Extracts from *Dunaliella tertiolecta, Rivularia firma* and *Gomphosphaeria aponina* have been reported to produce a range of pharmacological activities (Pabon de Majid and Martin, 1983; de Pauw and de la Noue, 1986; Borowitzka, 1988; Regan, 1988). However, the cyanobacterium *Lyngbya majuscula* is known to produce a toxin that is the causative agent of 'swimmer's itch' (Cohen, 1986). Clearly, as with all novel food preservatives, potential toxicological effects should not be ignored.

The major obstacle to the exploitation of algal products is the difficulty in adapting these marine plants and micro-organisms to mass cultivation methods. Microalgae are much more amenable than macroalgae to strictly controlled mass culture conditions and can be manipulated to produce the desired product in abundance. Thus, a potentially interesting biological activity will only be useful if access to future supplies of the same organism is assured. Several biotechnology companies in California, Australia, Israel and the UK are known to be working in this area.

Constraints

A number of factors can be expected to impede the rapid development of biotechnological production methods in the food industry in the near future. First, most foods are of a very complex nature and the interrelationships between the molecular components in food are still poorly understood. Consequently, the relationship between the functional properties of a food ingredient tested in isolation and the performance of that ingredient in the finished food product is often difficult to establish. Thus, an antimicrobial that works well *in vitro* will not necessarily perform well in a food and *vice versa*.

Secondly, there is often a weak relationship between the market value of a

food ingredient and its technical quality. Food is an emotional issue, which affects everyone personally. The properties of appearance, taste, freshness, texture, odour, 'naturalness', convenience, etc. are all judged subjectively by the consumer. Consumer acceptance of biotechnologically produced food preservatives will be critical in the success of these new products. In this respect, the association in the public mind of genetic engineering with biotechnology has already had adverse effects on the penetration of biotechnology in the food industry. In the future, it will be important to maintain an open flow of information between the food industry, health educators, consumer organizations and the media in order to avoid a reaction of the sort recently experienced with the introduction of food irradiation into the UK.

Thirdly, the regulatory clearances necessary for the manufacture of any new additive frequently involve the investment of substantial resources (Korwek, 1987; McNamara, 1989). The requirements for toxicological testing of new food ingredients are now so stringent that some food manufacturers are avoiding any new developments. Furthermore, many countries now require that a case of 'need' for any new additive be established. Nevertheless, precedents are being set with several biotechnologically derived products entering the market in 1989 and 1990: FDA approval for use in selected foods of gellan gum from *Pseudomonas elodea*; the mammalian chymosins from recombinant *Kluyveromyces lactis* and *E. coli*; and a genetically engineered bakers' yeast. All of these events will undoubtedly help to make the biotechnological production of food ingredients more acceptable to the public in the future.

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