Opinion on Riboflavin as a colouring matter authorized for use in foodstuffs produced by fermentation using genetically modified *bacillus subtilis* (expressed on 10 december 1998)

Terms of Reference

To advise on the safety in use of riboflavin, produced by fermentation using genetically modified *Bacillus subtilis*, as a colouring matter authorized for use in foodstuffs intended for human consumption.

Introduction

The Committee has previously reported on the safety in use of riboflavin and its metabolite riboflavin-5'-phosphate as a colouring matter for foodstuffs for human consumption (1). It has now been requested to review the microbiological and toxicological safety of riboflavin produced by fermentation as a colouring matter for foodstuffs generally.

Background

Riboflavin (vitamin B_2) is a water-soluble vitamin, that is synthesised by plants and many microorganisms but is not produced by higher animals. It is an essential micronutrient in the human diet, where it serves as precursor to coenzymes e.g. flavine adenine dinucleotide and flavin mononucleotide, which function as hydrogen carriers in biological redox processes.

Riboflavin occurs naturally in peas, beans, grains, yeast, milk, egg yolk and liver. Hitherto riboflavin has been chemically synthesised for use in food fortification and in small amounts as a colouring agent in foods e.g. ice cream, processed meat, fish products, sauces and soups. Riboflavin is also used in the fortification of animal feedstuffs.

Although it has been known for many years that riboflavin could be produced by bacteria using fermentation technology, it was not until recently that a very pure product could be produced using a genetically modified strain of *Bacillus subtilis*. The pathway of biosynthesis of riboflavin in *B. subtilis* involves 6 steps controlled by enzymes and the precursors guanosine triphosphate and ribulose-5-phosphate.

To evaluate the possible microbiological risks for consumer health the microbiological status of the production process requires consideration with regard to the safety of the producer organism as well as the fermentation process and product purification procedure. Although riboflavin is not a Novel Food per se the safety assessment can be performed following the strategy proposed by the SCF in its Guide to the Assessment of Novel Foods (2), particularly with reference to the section on Novel Foods derived from genetically modified microorganisms.

Producer organism

B. subtilis is an aerobic endospore-forming bacterium commonly found in nature and generally not considered to have a pathogenic or toxigenic potential. There is a history of safe use of this bacterium in large-scale fermentation production of specialty chemicals, of enzymes used in food production processes, and of several traditional relationships to food. It is used traditionally in East Asia for the fermentative production of Natto from wheat starch, a product also obtainable in western countries. It is thus an organism with a tradition in food use although the actual food product is little known in the European Union. The organism is also involved in many types of food spoilage.

The strain of *B. subtilis* used in riboflavin production is RB50::[pRF69] $_n$[pRF93] $_m$.Ade+ (3), a non-spor ing derivative of *B.subtilis* 168, which had been produced by classical mutation and into which the two specific plasmids pRF69 and pRF93 had been chromosomally, and thus stably, integrated into the bacterial genome and amplified 20-25-fold and 10-15-fold respectively. The strain was identified by the Deutsche Sammlung von Mikroorganismen (DSM Braunschweig, Germany) as *Bacillus subtilis*. The genetic modification includes the chromosomal integration of the following elements:

- pUC 19, a derivative of the common *E. coli* cloning vector pBR 322, harbouring a DNA fragment carrying the *rib* operon and the *bla* gene encoding ampicillin resistance, which latter is not expressed in *B. subtilis*.
- SPO1-15, the constitutive promoter derived from a *B. subtilis* bacteriophage which enables efficient expression of the *rib* operon.
- the marker gene *tet*, encoding tetracycline resistance, originally derived from *Bacillus cereus*.
- the marker gene *cat*, encoding chloramphenicol resistance, originally derived from *Staphylococcus aureus*.

The *tet* as well as the *cat* gene can be considered to be constituents of the natural gene pool of a wide range of Gram-positive organisms including *B. subtilis*. The chromosomal organisation of the gene inserts has been demonstrated by their functionality as well as by southern blot analysis. These data were supplemented by sequencing of specific fragments.

The fermentation process

The producer organism is grown by controlled submerged growth in well-defined media containing accepted components such as carbohydrates, nitrogen sources, mineral salts, an antifoam agent and the antibiotics chloramphenicol and/or tetracycline in the inoculation media.

The production process uses inoculum build-up followed by a feed-batch production fermentation. It is carried out in contained vessels using axenic large-scale microbiological techniques. The fermentation is routinely tested to ensure cultural purity, the quality control as described can be considered to be of the highest standard to exclude contamination. The final fermentation process will be performed in practice in compliance with Good Industrial Large-Scale practice.

Down-stream processing

After fermentation the whole broth is pasteurised to ensure that no viable cells of the production organism are present in the final product. The further processing is designed to remove cell debris, DNA and contaminants. Hydrochloric acid is added as the only purification agent. The concentration of acid and the temperature conditions chosen were shown to degrade any DNA still present, so that it can no longer serve as a substrate in transformation or as a template in a polymerase chain reaction. After removal of any residual biomass the purity of the final product is 96% and this material after washing and drying can then be used as such in animal feed. Further processing with hot hydrochloric acid produces a material of 98% purity (food grade). This final purification step is identical with that currently used for producing a chemically synthesised material of the same 98% purity. Comparison of the HPLC chromatograms of the chemically synthesised and the fermentation product confirmed that no further impurities had resulted from the fermentation process, the main degradation product resulting from the acid treatment being 8-hydroxymethylriboflavin (4). The final product meets the specification for riboflavin used as colouring matter for foodstuffs for human consumption listed in Commission Directive 95/45 EC (3).

Intake

The riboflavin produced by fermentation is intended to be used for the same purposes as the chemically synthesised product. No increased consumption is expected as no new applications exclusive to the fermentation-derived product are anticipated.

Nutritional aspects

As the synthetic product and the fermentation product have identical specifications and quality criteria and the same application levels, it is unlikely that there will be any difference in bioavailability, when one product is substituted for the other. Hence a nutritional assessment is unnecessary.

Toxicological aspects

The 96% fermentation product (feed grade), the 98% fermentation product (food grade) and the 98% chemically synthesised product were examined in a 13-week oral feeding study in Wistar rats at doses of 20, 50 or 200 mg/kg b.w. There were no significant differences between test and control groups regarding clinical signs, feed consumption and water intake. There was a 6% retardation of growth of females given 200 mg/kg b.w. of the 98% fermentation product. No consistent or dose-related changes in haematological parameters, urinalysis or clinical chemistry were noted. Females given 200 mg/kg b.w. of the 98% fermentation product showed a slight increase in relative liver weight and males given 50 and 200 mg/kg b.w. fermentation product showed a slight increase in relative spleen weight, neither of these weight increases being dose-related nor associated with any histopathological findings. Gross and histopathology showed no significant treatment-related lesions in any test group. The observed changes were considered to be of no toxicological concern (5)

A bacterial microsomal reverse mutation test in *S. typhimurium* was negative with and without S9 mix over a dose range of 50-5000 µg/plate for the 96% and 98% fermentation product (6,7)

Evaluation

The only genetic modifications of the producer organism *B .subtilis* concerned the introduction of the 2 genetic constructs containing the *rib* operons, the *tet* and *cat* genes and the selected mutational changes for deregulating the purine and riboflavin pathways and also for preventing sporulation. PCR analysis, southern blot analysis and sequencing of specific fragments showed that the expected composition of the modified genome remained stable under production conditions and that biologically active DNA of the production strain was no longer present in the fermentation-derived purified material. A hypothetical genetic transfer to the human consumer or the gut microorganisms of the novel genes in GM *B. subtilis* following consumption of fermentation-derived riboflavin could not occur since the purification process destroyed any DNA that may have been present.

There are no indications that the impurities present in the fermentation-produced riboflavin were different from those occurring in the chemically synthesised riboflavin and were also shown with existing analytical methods to be present at similar levels.

The toxicity study and the mutagenicity assay showed no toxicologically relevant adverse effects either in the 98% fermentation product or the 98% synthetic material. On microbiological-hygienic grounds there are no objections to the acceptance of the fermentation product.In conclusion the Committee considered that riboflavin produced by fermentation using genetically modified *Bacillus subtilis* is acceptable as a food colour.

References

1. SCF (1977) Fourth series of Reports, European Commission, Luxembourg
2. SCF (1997) Fourtieth series of Reports, European Commission, Luxembourg
3. Advisory Committee on Novel Foods and Processes (1996) Annual Report, UK
4. Simon, Vogt (1989) Roche Research Report BS/GCR 62589 submitted to the EuropeanCommission
5. Buser, Hofmann et al. (1995) Roche Research Report B 161165 submitted to the European Commission
6. Albertini, S. (1995) Roche Research Report B 164571 submitted to the European Commission
7. Albertini, S. (1995a) Roche Research Report B 164572, submitted to the European Commission