EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions
C3 - Management of scientific committees II; scientific co-operation and networks

SCIENTIFIC COMMITTEE ON FOOD

SCF/CS/ADD/AMI 52 Final
13 July 2000

Opinion of the Scientific Committee on Food on

ß-cyclodextrin produced using cycloglycosyltransferase from a recombinant *Bacillus licheniformis*

(Adopted by the SCF on 22/6/2000)

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Opinion of the Scientific Committee on Food on ß-cyclodextrin produced using cycloglycosyltransferase from a recombinant *Bacillus licheniformis*

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Terms of Reference

To evaluate the safety-in-use of ß-cyclodextrin as carrier and stabiliser manufactured by the action of the enzyme cycloglycosyltransferase obtained from a recombinant strain of *Bacillus licheniformis* when used as a food additive.

Background

ß-cyclodextrin (BCD) is a food additive with application as a carrier and stabiliser of food flavours, food colours and some vitamins. It is a cyclic heptamer composed of seven glucose units joined "head-to-tail" by α-1,4 bonds. It is employed in food as a flavour protecting agent at levels not exceeding 1 g/kg in food.

In this submission the petitioner proposes manufacture of BCD by a method using the enzyme cycloglycosyltransferase (CGTase) n°: 2.4.1.19 derived from a recombinant strain of *Bacillus licheniformis* (1).

The safety-in-use of the BCD manufactured by the action of CTGase obtained from *Bacillus circulans* on partially hydrolysed starch has already been evaluated by the SCF in 1996 when BCD was allocated an ADI of 0-5 mg/kg b.w.(2). The safety-in-use of a similar product manufactured by the action of CTGase obtained from *Bacillus macerans*, now known as *Paenibacillus macerans*, on various carbohydrates has been evaluated by JECFA in 1993 (3) and in 1995 (4).

Evaluation

The CGTase production process consists of a submerged culture fermentation using the recombinant producer organism *Bacillus licheniformis* strain SJ1608, the stock culture and fermentation culture both being controlled frequently for identity of the organism, absence of contaminating microorganisms, and enzyme yield before harvesting the enzyme. The purification process for the enzyme uses flocculation, ultrafiltration, evaporation, resolubilisation after precipitation and filtration through a microbiological filter. The final enzyme preparation has a purity of 99.0%-99.6%, contains no viable organisms of the recombinant producer strain, contains no detected carry-over DNA, and complies with the JECFA specification for CGTase.

The BCD is produced by the action of the recombinant CGTase on a carbohydrate substrate. Only limited information was supplied on the conditions for the production process in which the BCD is precipitated from the reaction mixture as a toluene complex, removed by ultracentrifuging, and eventually recovered after steam distillation, concentration, crystallisation and drying as a crystalline product. Any contaminating protein carried over by

the CGTase would be found only in the initial aqueous phase, from which the BCD-toluene complex has been separated. Any contaminating protein traces on the BCD crystals would be denatured by the subsequent heat treatment, the carbon decolourisation and the drying of the final BCD crystals, thus making them biologically inactive.

The batch used in the toxicological investigation of the CGTase was checked for the presence of live producer organisms and of the DNA of the recombinant strain by dot blot hybridisation analysis, the detection limit being 0.1 ng DNA (plasmid)/g It was further checked for any transferable DNA of *Thermoanaerobacter ATCC 53627* and of *Bacillus licheniformis* DN2717 by transformation assays capable of detecting any transformations in 50 mg samples. No recombinant or any other DNA was detected (9) and therefore transmission of any DNA to the final crystalline BCD is highly unlikely.

The absence of pathogenicity and toxigenicity of *B. licheniformis*, whilst not being absolutely demonstrated, can be considered assured by the absence of any reaction of the enzyme with antibody raised against enterotoxins produced by certain wild strains of *B. licheniformis* (9) and also by the negative results of i.p. challenge of mice with other recombinant *B. licheniformis* strains (9). There is furthermore a long history of safe use of these parent strains in food production.

The strain used for the production of the CGTase is *Bacillus licheniformis* SJ1608. The *apr* gene from *B. licheniformis* DN 2717 was cloned into a *Dra*1 fragment of *E. coli* K-12 and this was transformed into plasmid pE194 of *Bacillus subtilis* to form plasmid pPL1500 carrying kanamycin, and erythromycin resistance. The *cat* gene encoding chloramphenicol acetyl transferase was inserted into the *Sal*1 site in the *apr* gene of plasmid pPL1500, thus conferring additional chloramphenicol resistance and resulting in plasmid pPL1842. This plasmid was introduced into *B. licheniformis* DN2717 but was subsequently removed, leaving a chromosomally located *apr* gene interrupted by a *cat* gene in this strain. From this strain the *B. licheniformis* PL1980 strain was isolated by selection for chloramphenicol resistant, kanamycin sensitive colonies (4).

The *cgt*A gene encoding CGTase was derived from *Thermoanaerobacter* ATCC 53627 and cloned into *E. coli* K-12, while the *amy*L gene derived from *B. licheniformis* ATCC 9787 was cloned into *Bacillus subtilis*. In this latter organism gene fusion was performed between *amy*L expression signals and the *cgt*A sequence thus constructing plasmid pSJ 1359 which now contains the DNA of *Thermoanaerobacter* and the DNA of *Bacillus licheniformis* . The *amy*L-*cgt*A fusion gene is transferred to a pE194 derivative resulting in plasmid pSJ 1391 which was then integrated into the *B. licheniformis* strain PL1980 at PamyL with subsequent excision of the plasmid, so that the *amy*L-*cgt*A fusion gene remained in the chromosome. From this *B. licheniformis* strain PL1980 all other plasmids except the *amy*L-*cgt*A fusion gene were removed resulting in the formation of the production strain, *B. licheniformis* SJ1608. The only resistance marker retained was chloramphenicol resistance (4).

As the final crystalline BCD contains no detectable DNA or foreign protein and the CGTase itself was free of any transferable DNA no hazard from possible transferable antibiotic resistance through the use of BCD in food arises and therefore the presence of these marker genes in the recombinant producer organism for the CGTase is immaterial for safety consideration of the final BCD product

The final BCD is recovered as a crystalline preparation. Comparison of the HPLC chromatograms of the mother liquor of the recombinant CGTase and the mother liquor of the *B. circulans* CGTase showed the same patterns (4). Batch analysis showed it to contain some 0.07%-0.08% α-CD, some 0.04%-0.08% γ-CD and about 1% reducing sugars, thus complying with the JECFA specification for BCD prepared by the use of CGTase derived from *Bacillus circulans.*

Taking into account the purity of 99.6% of the CGTase, the purity of >99% of the BCD crystals, and that there are several purification steps included in the production processes of both the enzyme and the final BCD, the Committee considers this to constitute a dilution factor of at least six orders of magnitude for any *Bacillus* toxins possibly elaborated by the producer organisms. The Committee therefore considers it unnecessary to subject the final crystalline BCD to the additional analytical examination required to evaluate the safety of use of *Bacillus* species in animal nutrition as set out in the SCAN opinion (10).

The toxicity of the recombinant CGTase from *B. licheniformis* was examined in a 13-week gavage study in rats (5), in a test for gene mutation in bacteria (6), in a chromosomal aberration test in human lymphocytes (7) and in an assay for gene mutations in mouse lymphoma cells L5178Y (8). In none of these investigations were any treatment-related adverse effects detected.

There are no considerations relating to intake and nutritional aspects specifically needed for BCD made by fermentation with recombinant CGTase, which have not already been taken into account in the safety evaluation by the SCF for the BCD made by fermentation using the *B. circulans* CGTase as set out in the SCF Opinion of 1996 (1).

Conclusion

The Committee considers that BCD produced by the use of the CGTase derived from the recombinant *Bacillus licheniformis* strain SJ1608 is comparable with the BCD produced by CGTase derived from *Bacillus circulans* and complies with the existing JECFA specification for BCD. The Committee has no objection to the use as a food additive of BCD manufactured by the action of CGTase obtained this new method.

The BCD so produced should be included in the ADI of 0-5 mg/kg bw set earlier by the Committee for BCD produced by CGTase derived from *Bacillus circulans.*

References

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