Toxicological studies for food enzyme dossiers

Genotoxicity testing approach for the safety evaluation of food enzymes

Food Enzymes Team
FIP Unit
In accordance to Regulation (EC) No 1331/2008 and its implementing Rules, this new guidance describes the scientific data to be included in:

- applications for the authorisation of food enzymes,
- for the extension of use for existing authorisations.

**STRUCTURE OF THE GUIDANCE**

1. Source of the food enzyme
2. Production of the food enzyme
3. Characteristics of the food enzyme
4. **Toxicology**
5. Dietary exposure estimation

On the basis of the submitted data, EFSA will assess the safety of food enzymes and conclude whether or not they present risk to human health under the proposed conditions of use.

Toxicological studies are required for all food enzymes unless specifically exempted.

The studies should be performed according to OECD TG and following Good Laboratory Practice (GLP), or comparability of the applied guidelines should be provided along with a statement of GLP compliance of the laboratory conducting the study.

The most recent version of any guideline should be applied.
I. EFSA GUIDANCES ON MIXTURES

II. EFSA GUIDANCES ON GENOTOXICITY ASSESSMENT and TESTING STRATEGY (A STEP-WISE APPROACH)

III. SCIENTIFIC GUIDANCE FOR THE SUBMISSION OF DOSSIERS ON FOOD ENZYMES, EFSA CEP Panel, endorsed 25.2.2021
   - Recommended *in vitro* and follow-up *in vivo* genotoxicity tests
   - OECD Guidelines for the testing of chemicals
The concentrations/doses chosen for genotoxicity tests should be based on the amount of Total Organic Solids (TOS).

TOS may be calculated as $100\% - \% \text{water} - \% \text{ash} - \% \text{total added organic excipients}$ as defined by JECFA (FAO/WHO, 2006).

JECFA (FAO/WHO, 2006):
- To distinguish the proportion of the enzyme preparation derived from the source material and manufacturing process from that contributed by intentionally added formulation ingredients, the content of TOS is calculated.
- TOS is the sum of the enzyme protein together with other carryover material.

“MixTox Guideline” (EFSA SC, 2019):

➢ **MIXTURE**: Any combination of two or more chemicals that may contribute to effects regardless of source and spatial or temporal proximity.

➢ Based on this guidance on mixtures, a differentiation is made between:
  - mixtures that are chemically fully defined or characterized,
  - mixtures in which not all of the components have been characterized.

Component-based approach: the risk of combined exposure to multiple chemicals is assessed based on exposure and effect data of its individual components.

The whole mixture approach (if a mixture is poorly defined): the whole mixture is essentially evaluated in the same way as for a single chemical substance, and so requires dose–response information for the mixture of concern.

4.3 Genotoxicity

- Food enzymes are complex mixtures of unidentified components except the declared enzyme(s).

- The recommended approach for the genotoxicity assessment of such a type of mixture is to **test the whole mixture** (EFSA SC, 2019).

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FIRST A MIXTURE SHOULD BE CHEMICALLY CHARACTERISED AS FAR AS POSSIBLE, IF (EFSA SC, 2019):

- contains 1 or more chemical substances that are individually assessed to be genotoxic \textit{in vivo} via a relevant route of administration
- a fully chemically defined mixture does not contain genotoxic chemical substances
- contains a fraction of chemical substances that have not been chemically identified

- the mixture raises concern for genotoxicity
- the mixture is of no concern with respect to genotoxicity
- experimental testing of the unidentified fraction should be considered as the first option or, if this is not feasible, \textit{testing of the whole mixture} should be undertaken

set of \textit{in vitro} assays

THE GENOTOXICITY TESTING STRATEGY FOR A WHOLE MIXTURE

- should follow the SC testing strategy guidance for individual chemical substances
- (EFSA SC, 2019)

CLEARLY NEGATIVE RESULTS

set of in vitro assays

1 or more positive results

an in vivo follow-up study

expert judgement on the reliability of the results obtained (consideration of target tissue exposure)

NEGATIVE

no concern with respect to genotoxicity

POSITIVE

the mixture does raise a concern about genotoxicity

Demonstration of target tissue exposure in *in vivo* studies, particularly in the mammalian erythrocyte micronucleus test (OECD TG 474), selected as the appropriate follow-up of a positive *in vitro* outcome (aneugenicity/clastogenicity) (EFSA SC, 2017)

**Lines of evidence:**
Toxicity to the bone marrow in itself provides sufficient evidence to allow concluding on the validity of a negative outcome of a study:
- in the MN test (*a decrease in the PCE/(NCE+PCE) ratio*);
- in toxicity studies (*a histopathological change in the bone marrow* in such a study using the same route and the same species as in the MN test, if evaluated as being test-substance related).

**Other lines of evidence that should be assessed within a Weight of Evidence approach:**
- *test substance/metabolites detected in the bone marrow* in a toxicokinetic study (OECD TG 417) (detection of radioactivity in the bone marrow, using the same route and the same species);
- *systemic toxicity* observed in MN test (clinical signs, i.e. related to the CNS)/or in toxicity studies;
- *test substance/metabolites detected systemically in a toxicokinetic study* (detection of radioactivity in the blood/plasma, organs, tissues, urine in an ADME study using the same route and the same species)/or *in a specific blood/plasma analysis.*
Basic battery of *in vitro* tests:
- Bacterial reverse mutation assay (OECD TG 471)
- *In vitro* micronucleus assay (OECD TG 487)

If one or two tests are POSITIVE, consider the following for *in vivo* follow up:
- *In vivo* mammalian erythrocyte micronucleus test (OECD TG 474)
- Transgenic rodent cell gene mutation assay (OECD TG 488)
- *In vivo* Comet assay (internationally agreed protocols, OECD TG 489)

The *in vivo* test selected should relate to the genotoxic endpoint(s) identified as positive in the *in vitro* tests.

If all *in vitro* endpoints are clearly NEGATIVE, no genotoxic potential

If the *in vitro* tests are INCONCLUSIVE, CONTRADICTORY or EQUIVOCAL, further *in vitro* tests to clarify the genotoxic potential *in vitro*

If *in vivo* NEGATIVE, no genotoxic potential

If *in vivo* POSITIVE, genotoxic potential

Fig. 1: Schematic representation of genotoxicity testing strategy recommended by the EFSA SC (2011)
GENOTOXICITY TESTING APPROACH FOR THE SAFETY EVALUATION OF FOOD ENZYMES

III. SCIENTIFIC GUIDANCE FOR THE SUBMISSION OF DOSSIERS ON FOOD ENZYMES, EFSA CEP Panel, endorsed 25.2.2021

- Recommended in vitro and follow-up in vivo genotoxicity tests

Guidelines for the testing of chemicals
The combination of two *in vitro* tests, recommended as the first step (EFSA SC, 2011), fulfills the basic requirements to cover the three genetic endpoints with the minimum number of tests:

- bacterial reverse mutation assay (OECD TG 471) - covers gene mutations

- *in vitro* mammalian cell micronucleus test (OECD TG 487) - covers both structural and numerical chromosomal aberrations
In order to overcome potential problems with histidine or tryptophan in the food enzyme batch, it is recommended to expose the *Salmonella* and *E. coli* strains to the tested food enzyme in the liquid culture ("treat and plate assay"), instead of the traditionally "plate incorporation assay".

A recommended protocol is given in Annex D.

The recommended maximum test concentration for soluble non-cytotoxic substances is at least 5 mg TOS/plate. This concentration is necessary to ensure sufficient level of exposure to detect the majority of known genotoxic compounds.
The highest test concentration should correspond to 2 mg TOS/mL, if no precipitate or limiting cytotoxicity is observed.

However, the top concentration may need to be higher than recommended, e.g., up to 5 mg TOS/mL, to increase the concentration of each of the components in the absence of sufficient cytotoxicity (OECD TG 487) and EFSA SC (2019).
In vivo follow-up, in line with the recommendation of the EFSA SC (2011; 2017), for substances positive in the in vitro basic battery:

- *in vivo* mammalian erythrocyte micronucleus assay for *in vitro* clastogens and aneugens (OECD TG 474);

- *in vivo* mammalian alkaline comet assay for substances which cause gene mutations and/or structural chromosomal aberrations (OECD TG 489);

- transgenic rodent gene mutation assay to follow-up *in vitro* positive compounds for gene mutations (OECD TG 488);

- a combination of an *in vivo* micronucleus assay and a Comet assay in the event of a positive *in vitro* micronucleus assay.

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EFSA SC, 2011. Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA J 2011;9(9):2379. 69 pp.;