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Short communication

Potential pitfalls associated with testing of enzyme preparations in the *Salmonella*/microsome assay

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ABSTRACT

The effect of a sample of food enzyme preparations on S9 activity was evaluated in bacterial mutation assays with the *Salmonella typhimurium* strains TA98 and TA100 using benzo(a)pyrene, 2-aminoanthracene and 2-aminofluorene as model compounds. Under the experimental conditions applied, *Aspergillus oryzae* protease and porcine pancreas trypsin, applied at low non-toxic doses, proved to effectively inhibit the metabolic activation of benzo(a)pyrene by Aroclor induced rat liver 9, while the activation of 2-aminoanthracene and 2-aminofluorene was only marginally affected. The tolerance of metabolic activation of 2-aminoanthracene to the presence of proteolytic enzymes, compared to the strong inhibition elicited on the metabolic activation of benzo(a)pyrene, points to the involvement of different components of liver S9 in their biotransformation. Overall, data indicate that the use of 2-aminoanthracene as positive control in the Ames test can give a misleading indication of S9 proficiency, and thus it should be used with caution or in conjunction with other chemicals, especially in the testing of crude enzyme preparations in which proteases may be present as minor components.

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1. Introduction

Enzyme preparations are widely used in the manufacture, processing, treatment, packaging or storage of food items (European Union, 2008a). When highly purified, or obtained from edible plants or animals and associated with a history of safe use, the safety of food enzymes can be established with no need for further toxicity testing. In fact, because of the proteinaceous nature of food enzymes and their susceptibility to degradation by digestion, a limited toxicological potential is expected following oral exposure (EFSA, 2005; FDA, 2000). However, in most cases commercial enzyme products are only partially purified, and contain a variety of uncharacterized components of biological origin or resulting from the purification process. In these cases a toxicological evaluation may be required prior to their introduction on the market. Under the European food regulation, for example, a core set of toxicological studies covering both genotoxicity and systemic toxicity is required for inclusion of food enzymes in the Community positive list (European Union, 2008b; EFSA, 2009).

The base set of genotoxicity tests to be performed normally includes a test for induction of gene mutation in bacteria, *viz*., the Salmonella/microsome or Ames test, as described in the OECD guideline 471 (OECD, 2015). The Ames test pioneered in the 70s the development of short-term assays for the prediction of carcinogenicity, and still plays a key role in genotoxicity testing batteries (Eastmond et al., 2009) being the first, and sometimes the only (ECHA, 2015) mutagenicity test performed. However, testing of raw enzyme preparation in the Ames test may be problematic in case free amino acids are present, because of their feeding effect and the resulting overgrowth of the background lawn on histidine-requiring cells, which may hinder the effective selection and reliable identification of true histidine-independent revertant colonies (Aeschbacher et al., 1983; Nylund and Einisto, 1993). Thus, to avoid complications associated to the release of amino acids, alternative test methods, e.g. mutation tests in mammalian cells (EFSA, 2009), or modified test procedures, e.g. the “treat and wash” modified preincubation method (Thompson et al., 2005), have been recommended for testing of proteinaceous materials.

Beyond the undesirable presence of free amino acids, another potential problem associated with *in vitro* testing of enzymes concerns the interference of test material with the activity of the exogenous metabolic activation system. The latter, normally consisting of a rodent liver post-mitochondrial fraction (S9), roughly mimics mammalian metabolism of xenobiotics *in vitro*, and plays a

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key role in the Ames test enabling the effective detection of most chemical carcinogens (Ames et al., 1973; McCann et al., 1975). Indeed, the possibility that the enzyme preparation tested elicited some subsidiary/side activity (e.g. protease, phospholipase) affecting the activity of liver S9 was previously flagged, with the recommendation to add the enzyme preparation also to the positive control in order to demonstrate that the S9 activity was not affected (EFSA, 2014).

Concerning positive control, according to the OECD TG471 several options can be considered, depending of the bacterial strain. Among the positive control substances to be used with metabolic activation, the aromatic amine 2-aminoanthracene is most frequently used, as relatively stable in solution and highly effective with most tester strains. However, as both cytosolic and microsomal enzymes concur to the metabolic activation of 2-aminoanthracene, the OECD TG471 recommends that, when 2-aminoanthracene is routinely used, the S9 batch be also characterized with a mutagen specifically requiring activation by microsomal enzymes, such as benzo(a)pyrene (OECD, 2015).

In this work we have further explored the effect of a few food enzyme preparation on the *in vitro* metabolic activation of benzo(a)pyrene and two aromatic amines, 2-aminoanthracene and 2-aminofluorene, also in relation to their suitability as controls of S9 efficiency in the testing of food enzymes.

2. Materials and methods

2.1. Chemicals

2-aminofluorene [153-78-6] and benzo(a)pyrene [50-32-8] were purchased from Sigma-Aldrich Italia (Milan, It). 2-Aminoanthracene [613-13-8] was a gift of Dr. J. Ashby, Macclesfield, UK. Spectrophotometric grade dimethylsulfoxide (DMSO), used as solvent, was from Carlo Erba Reagents (Milan, It). Protease (from *Aspergillus oryzae*), lipase (from *Aspergillus niger*), glucose oxidase (from *A. niger*), xylanase (from *Thermomyces lanuginosus*) were from Sigma-Aldrich Italia (Milan, It), trypsin (from porcine pancreas) was from Gibco™ (Thermo Fisher Scientific Inc., USA).

2.2. Bacterial mutagenicity assays

The *Salmonella typhimurium* strains TA98 and TA100, provided by MolTox Inc. (Boone, NC), were used throughout the work. Plate incorporation and pre-incubation tests were carried according to the OECD Test Guideline 471 (OECD, 2015): for the plate incorporation assay, bacteria, S9mix, reference mutagen and/or enzyme preparation, as appropriate, were directly added to top agar and poured onto agar plates. For the pre-incubation method, test tubes with bacteria, test substances and metabolic activation system were pre-incubated for 20 min at 37 °C with gentle shaking prior to mixing with the overlay agar.

A modified pre-incubation method, thereafter defined "treat and wash", was also applied following the procedure originally described by Thompson et al. (2005). Briefly, the pre-incubation time was increased to 90 min, afterwards bacteria were washed in nutrient broth:saline (1:7), collected by centrifugation and plated as usual.

S9 mix containing 10% of Aroclor-induced rat liver S9, provided by Trinova Biochem (Giessen, DE), and the appropriate cofactors mix [11], was used as exogenous metabolic activation source in all assays. The microsomal activity of the S9 batch was preliminary assessed by the manufacturer with a range of standard mutagens, which included benzo(a)pyrene and 2-aminoanthracene.

Stock solutions in DMSO of the positive control substances, aliquoted and stored at −20 °C, were used throughout the work. Enzyme preparations were diluted or dissolved in saline and stored at 4 °C before testing. Fresh enzyme solutions were prepared weekly.

All the results were verified and confirmed in repeated experiments, usually adjusting the experimental conditions (treatment time, enzyme or mutagen dose) in order to get the optimal response. For the sake of clarity and conciseness, only the results of experiments with optimized conditions are reported.

3. Results

Range-finding experiments were preliminary carried out with all enzyme preparations using the pre-incubation procedure, in order to identify a proper sub-toxic dose range for main experiments.

No genotoxic effect, and no inhibition of the metabolic activation of benzo(a)pyrene, 2-aminofluorene and 2-aminoanthracene, was observed in pre-incubation experiments with the food enzymes *A. niger* lipase (1–3 U/plate, highest non-toxic concentration) and *T. lanuginosus* xylanase (up to 12.5 U/plate, limit of solubility in agar). *A. niger* glucose oxidase proved to be exceedingly toxic when incorporated in top agar (either in the plate incorporation and pre-incubation assays), completely inhibiting bacterial growth from 0.5 U/plate onwards. Further experiment using the "treat and wash" procedure, in which cytotoxicity was less prominent, indicated also for glucose oxidase lack of genotoxicity and inhibition of S9 activity in the dose range tested (1–3 U/plate). The results of typical experiments with lipase, xylanase and glucose oxidase are reported in Appendix A (Supplementary Data).

A.oryzae protease elicited a dose-related toxicity, with a progressive inhibition of bacterial growth from 10 U/plate onwards. At lower doses *A. oryzae* protease was not toxic but inhibited effectively the metabolic activation of benzo(a)pyrene to a mutagen in pre-incubation tests with *S. typhimurium* TA98 (Fig. 1). In the same experimental conditions *A. oryzae* protease also inhibited the mutagenicity of 2-aminofluorene and 2-aminoanthracene, although less effectively than for benzo(a)pyrene: at a low dose (0.25 U/plate) benzo(a)pyrene mutagenicity was largely (83%) inhibited, while the activities of both 2-aminofluorene and 2-aminoanthracene were unaffected (Fig. 2).

The inhibitory effect of *A. oryzae* protease on the metabolic activation of benzo(a)pyrene was further evaluated in strain TA100, in parallel assays using the plate incorporation and "treat and wash" procedures. In these experiments the inhibitory effect of *A. oryzae* protease on the metabolic activation of benzo(a)pyrene proved not to be mitigated by the "treat and wash" procedure, in which the effect of low doses of the enzyme preparation was rather

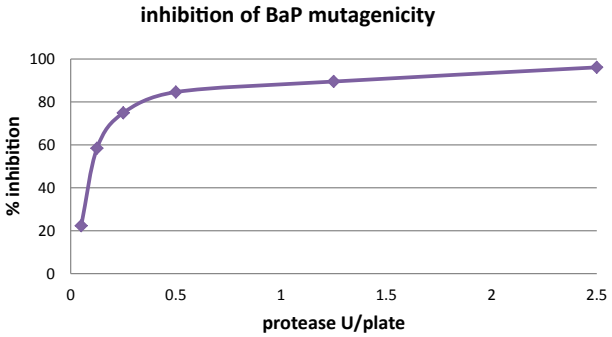

Fig. 1. Inhibition of benzo(a)pyrene mutagenicity by *A. oryzae* protease. Pre-incubation test with *S. typhimurium* TA98. Raw data of all figures are available as supplementary data in Appendix A.

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