10 March 2020
[117-20]

Supporting document 1

Risk and technical assessment – Application A1174

Xylanase from a GM *Trichoderma reesei* as a processing aid (enzyme)

Executive summary

The purpose of the application is to seek amendment of Schedule 18 to Standard 1.3.3 – Processing Aids of the Australia New Zealand Food Standards Code (the Code) to include the enzyme endo-1,4-beta-xylanase (xylanase[1]), produced by *Trichoderma reesei* genetically modified to express the endo-1,4-beta-xylanase gene from *Aspergillus niger* (var. *tubingensis*). The intended use of the enzyme is in the manufacture of bakery products and cereal-based products, including beverages.

The evidence presented to support the proposed use of the enzyme provides adequate assurance that the enzyme, in its recommended form and amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme meets international purity specifications.

No public health and safety concerns were identified in the assessment of endo-1,4-beta-xylanase produced from a genetically modified (GM) strain of *T. reesei*.

The endo-1,4-beta-xylanase that is the subject of this application has a history of safe use in other countries. Endo-1,4-beta-xylanase produced directly from *A. niger* is already permitted in the Code. *T. reesei* also has a history of safe use as the production organism for a number of enzyme processing aids that are permitted in the Code. The *T. reesei* production strain is neither toxigenic or pathogenic and is absent in the final enzyme preparation. Molecular characterisation of the production strain confirmed the inserted DNA is present and is stably inherited.

Analysis of the enzyme showed no evidence of genotoxicity in a bacterial reverse mutation assay or a chromosomal aberration assay. In a 90-day oral gavage study in rats, the No Observed Adverse Effect Level (NOAEL) was the highest dose tested, 1000 mg/kg bw/day total protein, which is equivalent to 1214 mg/kg bw/day total organic solids (TOS). The Theoretical Maximum Daily Intake (TMDI) was calculated by the applicant to be 0.635 mg/kg bw/day TOS. Comparison of the NOAEL and the TMDI gives a Margin of Exposure (MoE) of approximately 1900.

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[1] The application seeks permission for xylanase, but the accepted IUBMB name and the name used throughout this document (which also reflects the listing in the Code), is endo-1,4-beta-xylanase.

Bioinformatic data indicated a lack of homology of the enzyme protein with known toxins or allergens. The enzyme preparation contains wheat starch and wheat flour, and wheat bran and soy may be present due to their use as nutrients in the fermentation process to produce the enzyme.

Based on the reviewed data it is concluded that in the absence of any identifiable hazard an Acceptable Daily Intake (ADI) 'not specified' is appropriate. A dietary exposure assessment was therefore not required.

Table of contents

1 Introduction

Endo-1,4-beta-xylanase (EC 3.2.1.8) is an enzyme which catalyses the endohydrolysis of (1→4)-beta-D-xylosidic linkages in xylans. The enzyme is intended to be used in baking for the production of bread, buns, cakes, sweet goods, tortillas and various other bakery products and also for the manufacture of cereal-based beverages.

The enzyme is produced by fermentation from a genetically modified (GM) strain of *Trichoderma reesei* expressing the endo-1,4-beta-xylanase gene from *Aspergillus niger*.

1.1 Objectives of the assessment

The objectives of this Risk and Technical Assessment for endo-1,4-beta-xylanase were to:

- determine whether the proposed purpose is clearly stated and that the enzyme achieves its technological function in the quantity and form proposed to be used as a food processing aid

- evaluate any potential public health and safety issues that may arise from the use of this enzyme protein, produced by a GM organism as a processing aid. Specifically by considering the:

    – history of use of the host and gene donor organisms
    – characterisation of the genetic modification(s), and
    – safety of the enzyme protein.

2 Food technology assessment

2.1 Characterisation of the enzyme

2.1.1 Identity and properties of the enzyme

The production microorganism of the enzyme is a GM strain of *T. reesei*. The donor microorganism of the endo-1,4-beta-xylanase gene is *A. niger* (further details contained in section 3).

Generic common name:	Xylanase
Accepted IUBMB[2] name:	Endo-1,4-beta-xylanase
Systematic name:	4-beta-D-xylan xylanohydrolase
Other names:	endo-(1→4)-β-xylan 4-xylanohydrolase; endo-1,4-xylanase; xylanase; β-1,4-xylanase; endo-1,4-xylanase; endo-β-1,4-xylanase; endo-1,4-β-D-xylanase; 1,4-β-xylan xylanohydrolase; β-xylanase; β-1,4-xylan xylanohydrolase; endo-1,4-β-xylanase; β-D-xylanase
EC number:	3.2.1.8

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[2] International Union of Biochemistry and Molecular Biology

CAS[3] registry number:	9025-57-4
Reaction:	Endohydrolysis of (1→4)-β-D-xylosidic linkages in xylans
Optimal temperature, range	50°C, 45-55°C
Optimal pH, range	4.0, 3.5 – 4.5
Stability, enzyme preparation, 20°C	12 months

2.1.2 Technological purpose of the enzyme

The technological purpose of this enzyme is similar to that of other already permitted forms of the enzyme, in that it will be used in the manufacture of bakery products and cereal-based products, including beverages. In particular, the application provides examples supporting its use in the production of different bakery products such as breads, buns, cakes, sweet bakery products, tortillas and various other bakery and other cereal-based products including beverages, in particular the use in brewing.

The enzyme performs its technological function in the manufacture of bakery products during dough or batter handling, to improve dough stability and handling properties. It does this by partially hydrolysing (degrading) the hemicellulose network of the dough to produce lower molecular weight and more soluble products, which increases the water binding capacity and baking properties of the subsequent dough.

The enzyme can also perform a technological function in the manufacture of cereal-based beverages; essentially in the brewing process to produce such products.

2.1.3 Technological justification of the enzyme

Information was provided in the application (and the applicant's submission at the Call for Submission stage relating to its use in brewing and cereal-based beverage production) supporting the benefits of using the enzyme in the baking and brewing industries. The specific benefits are summarised in Table 1 (a) and (b) below.

Table 1   Technological justification and benefits of using the enzyme during food processing (baking and brewing)

(a) Baking

Area of use	*Benefit*
Dough processing	• Better handling, including improved extensibility and stability, reduced stickiness and hence reduced losses
Baking	• Improvement in dough structure and behaviour
Final baked product	• Slightly increased volume, more uniform and improved crumb structure
Batter processing	• Reduced viscosity, improvement for processing

(b) Brewing

Area of use	*Benefit*

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[3] Chemical Abstracts Service

Mashing step	• Increased flexibility in choice of grains, greater opportunity to use barley as alternative to malted barley when used in combination with beta-glucanase. • Better, more predictable and faster lautering (mash filtration) rate. • Higher extract yield, and less losses and lower viscosity due to reduction in high molecular weight beta-glucans. • Allows better access to the indigenous enzymes from the malt or barley to the starch granules and less losses of water to spent grains.
Fermentation	• Can also be added at this step. Again will perform a similar function to reduce levels of high molecular weight beta-glucans and improve efficiencies of filtration.

For brewing processing the enzyme can be added either during mashing, or to the sugar adjunct added to the mash, or during the fermentation step. In both cases subsequent heating steps will denature the enzyme; being the wort (the liquid filtered from mashing) boiling step, and pasteurisation respectively.

2.2 Manufacturing process

2.2.1 Production of the enzyme

The enzyme is produced by a submerged fermentation process, which is the common production method of producing food enzymes, as shown in Figure 1.

Fermentation
The fermentation process involves three steps: inoculation, seed fermentation and main fermentation.

Recovery
The recovery process is a multi-step operation designed to separate the enzyme from the microbial biomass and purify, concentrate, and stabilise the food enzyme.

The manufacturing process is such that the production microorganism, *T. reesei*, is removed during processing and so is absent from the commercial enzyme preparation.

The enzyme is standardised to ensure consistent enzyme activity and produced as a powdered enzyme preparation with wheat starch and wheat flour used as the carrier.

2.2.2 Specifications

There are international specifications for enzyme preparations used in food production. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2017) and the Food Chemicals Codex (FCC) (USP, 2018). Both of these specification sources are primary sources listed in section S3—2 of the Code. Enzyme preparations must meet these specifications.

Table 2 provides a comparison of representative batch analysis of the enzyme preparation with the international specifications established by JECFA and Food Chemicals Codex, as well as those detailed in the Code (being section S3—4, as applicable). Analytical results for heavy metals (lead, arsenic, cadmium and mercury) confirm that representative batches meet the requirements of S3—4 of the Code.

Table 2: Product specifications for commercial enzyme preparation

Analysis	Enzyme batch analysis	Specifications		
		JECFA	Food Chemicals Codex	Code
Lead (mg/kg)	<0.05	≤ 5	≤ 5	≤2
Arsenic (mg/kg)	<0.1	-	-	≤1
Cadmium (mg/kg)	<0.01	-	-	≤1
Mercury (mg/kg)	<0.01	-	-	≤1
Total coliforms (cfu/g)	<1	≤30	≤30	-
Salmonella (in 25 g)	negative	Absent	Negative	-
Enteropathic *E. coli* (in 25 g)	negative	Absent	-	-
Antimicrobial activity	negative	Absent	-	-

Based on the above results, the enzyme preparation meets international and Code specifications for enzymes used in food production.

2.3 Food technology conclusion

FSANZ concludes that the stated purpose of this enzyme preparation, namely for use as a processing aid in the manufacture of bakery products and cereal-based products including beverages, is clearly articulated in the application and subsequent submission. The evidence presented to support the proposed uses provides adequate assurance that the enzyme, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme performs its technological purpose during production and manufacture of foods after which it is inactivated thereby not performing a technological function in the final food. It is therefore appropriately categorised as a processing aid and not a food additive. The enzyme preparation meets international purity specifications.

3   Safety assessment

3.1 Objectives for safety assessment

The objectives of this safety assessment for endo-1,4-beta-xylanase are to evaluate any potential public health and safety concerns that may arise from the use of this enzyme protein, produced by a GM microorganism, as a processing aid. Specifically this will be by considering:

- history of use of the host and gene donor microorganisms
- characterisation of the genetic modification(s)
- safety of the enzyme protein.

3.2 History of use

3.2.1 Host organism

Trichoderma reesei is a hypercellulolytic fungus commonly found in soil. The initial isolate came from deteriorating clothing and tent material found in the Solomon Islands after World War II. The initial isolate (QM6a) has been registered with the American Type Culture

Collection as the type strain for the species (ATCC 13631). It has been classed as a Biosafety Level 1 organism—based on the United States Public Health Service Guidelines (U.S. DHHS 2009)—and is not considered pathogenic to humans. All strains of *T. reesei* used today in biotechnology have been derived from this particular isolate (Nevalainen et al 1994; Seidl et al 2009).

Due to the secretion of a range of cellulolytic enzymes, this fungus has been used since the 1980s for the industrial production of enzymes for a range of industries including food (Nevalainen and Peterson, 2014; Paloheimo et al, 2016). FSANZ has previously assessed the safety of *T. reesei* as the source organism for a number of enzymes used as processing aids. Schedule 18 to Standard 1.3.3 of the Code permits the use of the following enzymes derived from non-GM *T. reesei* strains: cellulose; endo-1,4-beta-xylanase, β-glucanase; hemicellulase multicomponent enzyme; and polygalacturonase or pectinase multicomponent enzyme. As part of FSANZ's assessment, the taxonomy of the host strain was verified.

3.2.2 Gene donor organism(s)

The gene sequence for the endo-1,4-beta-xylanase enzyme was sourced from *A. niger (*var. *tubingensis)*. This organism is a filamentous fungus, commonly found in soil and has been associated with food spoilage (Samson et al. 2007). There is some evidence of pathogenicity and toxin production for some strains of this fungus (Samson et al. 2006; Gautier et al. 2016). However, as the gene for this enzyme has been manipulated through standard DNA cloning methods subsequent to its isolation, extraneous material from *A. niger* would not be carried across to the enzyme production organism.

3.3 Characterisation of the genetic modification(s)

Full details of the genetic modification to the production organism were provided to FSANZ for assessment but cannot be disclosed as they are confidential commercial information. A summary of FSANZ's assessment of that information is given below.

3.3.1 Description of DNA to be introduced and method of transformation

A single expression cassette was generated and host transformation performed using standard methodologies. The expression cassette contained two coding regions. The first coding region contains the endo-1,4-beta-xylanase gene, flanked by well characterised promoter and terminator sequences. The second coding region contains a metabolic selective marker gene.

3.3.2 Characterisation of inserted DNA

Southern blot analyses were used to confirm presence of the inserted DNA in the hosts' genome, absence of the vector backbone sequences and to determine gene copy number.

3.3.3 Genetic stability of the inserted gene

The genetic stability of the inserted gene was investigated and no issues were identified.

3.4 Safety of endo-1,4-beta-xylanase

The enzyme that is the subject of this application meets the specifications of JECFA and the Food Chemicals Codex.

3.4.1 History of safe use of the enzyme

According to the applicant, this enzyme has been used for bakery applications in several countries, including Norway and Peru since 2016, as well as being considered GRAS (Generally Recognized as Safe) in the USA since 2015, without reports of adverse effects.

A number of other endo-1,4-beta-xylanases have been approved by FSANZ and are listed in Schedule 18 of the Code. These include one produced by *A. niger*. The enzyme that is the subject of the current application is produced in *T. reesei* genetically modified to express the endo-1,4-beta-xylanase of *A. niger*.

3.4.2 Bioinformatics concerning potential for toxicity

A BLAST search for homology of the endo-1,4-beta-xylanase sequence was performed against the complete Uniprot database (http://wwwuniprot.org/), using a threshold E-value of 0.1. A number of homologies were found, predominantly to other endo-1,4-beta-xylanases. None of the top 1000 matches were annotated as being either a toxin or a venom. A specific BLAST search for homology of the mature enzyme sequence was performed against the Uniprot animal toxin database, and yielded no matches. Therefore the endo-1,4-beta-xylanase sequence does not share significant homology with that of any known toxin or venom.

3.4.3 Toxicology studies in animals

90-Day oral gavage study of xylanase in Sprague Dawley rats (DuPont Haskell 2016). Regulatory status: GLP; conducted in compliance with OECD Section 4, Part 408

The test article for this study was the endo-1,4-beta-xylanase that is the subject of the application. The control article and vehicle was deionized water. Test subjects were Crl:CD(SD) rats. Rats were pair-housed in solid-bottom caging with bedding, under standard laboratory environmental conditions. Standard rat food was supplied *ad libitum*, except during scheduled fasts, and tap water was supplied *ad libitum*. After 7 days of acclimatization, and following baseline ophthalmological and neurobehavioural evaluations, rats were assigned, 10/sex/group, to dose groups, and dosing commenced when the rats were approximately 7 weeks old. Rats were dosed once daily by oral gavage with 0, 250, 500 or 1000 mg/kg bw/day for 90 days, in a dose volume of 12 mL/kg bw. Dosing formulations were prepared, stored and used within the pre-established conditions of stability, and sampled for concentration analysis in weeks 1, 6 and 11 of study. During the in-life phase, rats were observed twice daily for moribundity/mortality, and cageside clinical observations were made once daily. Detailed clinical observations, bodyweights and food consumption were recorded weekly. All rats were subject to ophthalmological examination on Day 86, and a neurobehavioural evaluation which was conducted on Day 89 or 90. The neurobehavioural evaluation included a motor activity assessment and an abbreviated functional observational battery. Overnight prior to scheduled termination, rats were fasted in metabolism cages with access to water, and urine was collected for at least 15 hours. Rats were anaesthetized with isoflurane and blood was collected for hematology and serum clinical chemistry. Rats were then killed by exsanguination and necropsied. Fresh organ weights were recorded for adrenal glands, brain, heart, kidneys, liver, spleen, thymus, gonads, uterus of females and epididymides, prostate, seminal vesicles and coagulating glands of males. A comprehensive list of organs, as well as gross lesions, were fixed. Tissues from control and 1000 mg/kg bw/day rats were processed for routine histopathological examination.

Dose formulations were within 10% of target concentrations, with the exception of the 250 mg/kg bw formulation in Week 11, which was 36% below target concentration. One 1000

mg/kg bw/day female was euthanased on Day 62 due to gavage error, but all other rats survived to the end of the in-life phase. There were no treatment-related effects on clinical observations, bodyweights, bodyweight gains, food consumption, food efficiency, ophthalmological findings, neurobehavioural findings, haematology, clinical chemistry, urinalysis, absolute or relative organ weights, gross findings on necropsy, or microscopic findings. It was concluded that the No Observed Adverse Effect Level (NOAEL) was the highest dose administered, 1000 mg/kg bw/day. Expressed as TOS, this is 1214 mg/kg bw/day.

3.4.4 Genotoxicity assays

Two genotoxicity studies were submitted, a bacterial reverse mutation assay (Ames test) and a chromosomal aberration test in human peripheral blood lymphocytes. Both were conducted using the endo-1,4-beta-xylanase that is the subject of this application as the test article.

Bacterial reverse mutation assay (BioReliance 2014). Regulatory status: GLP; conducted in compliance with OECD Section 4, Part 471, and with US FDA guidelines (Redbook 2000).

Test strains for this assay were *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and *Escherichia coli* strain WP2 *uvr*A. Almost all assays, in both the dose-ranging phase and the confirmatory phase were performed by the treat-and-plate method. This method was chosen because the test article potentially contains histidine, which could cause false positive results, and the treat-and-plate method includes a rinsing step that removes the test article. The exception was the positive control assay with 2-aminoanthracene (2AA), in the presence of S9 activation, with *E. coli* WP2 *uvr*A, which was performed by the plate incorporation method.

The concentrations of test article used in the dose-rangefinding assay were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg/plate. The vehicle and negative control article was sterile water. Positive control articles in assays without S9 were 2-nitrofluorene for TA98, ICR-191 for TA1537, and N-methyl-N-nitro-N-nitrosoguanidine for TA100, TA1535, and WP2 *uvr*A. The positive control for all bacterial strains in assays in which S9 was included was 2AA. S9 or sham mix, tester strain and vehicle, test article or control article were mixed in a test tube and incubated for 60±2 minutes at 37±2°C, with shaking. All assays in the dose-rangefinding phase were conducted in duplicate. At the end of incubation, the tubes were centrifuged, the supernatant was removed and the pellet, comprising tester bacteria, was resuspended. The resuspended bacteria were mixed with top agar, plated and overlaid on bottom agar, and the inverted plates were incubated for 48 to 72 hours at 37±2°C. Colony counting was then performed. No significant change in revertant counts was observed in the presence of the test article. Significant increases in revertant counts in the presence of the positive controls confirmed the validity of the assay. No precipitation of test article was observed. Evidence of toxicity on TA100 was observed with 5000 µg/plate, in the absence of S9, and the assay of the test article with TA100 was repeated at test article concentrations of 50, 150, 500, 1500 and 5000 µg/plate. Based on the results, 5000 µg/plate was selected as the highest dose for the confirmatory assay.

The confirmatory assay was conducted according to the same method as the dose rangefinding assay, but with test article concentrations of 50, 150, 500, 1500 and 5000 µg/plate. No precipitate or toxicity was observed. There was no evidence of mutagenic effects of the test article on TA100, TA1535, TA1537 or WP2 *uvr*A, with or without S9 mix. The assays with TA98 was repeated because of confluent bacterial colony growth, and showed no positive mutagenic response, with or without S9 mix. Positive controls induced significant mutagenesis, confirming the validity of the assay.

It was concluded that the test article did not show any mutagenic effects.

In vitro mammalian chromosome aberration assay in human peripheral blood lymphocytes (BioReliance 2014). Regulatory status: GLP; conducted in compliance with OECD Section 4, Part 473

The test system for this assay comprised human peripheral blood lymphocytes (HPBL) collected from a healthy non-smoking adult man.

A preliminary toxicity test was conducted by exposing cultured HPBL to the vehicle/negative control article, sterile water, or one of nine concentrations of test article, ranging from 0.5 to 5000 μg/mL with half-log dose spacing. Exposure was conducted in both the presence and absence of S9 mix for 4 hours, or continuously for 20 hours in the absence of S9 mix, during incubation at 37±1°C. Positive control assays were conducted in parallel, using Mitomycin C as the positive control for non-activated test system and cyclophosphamide as the positive control for the S9-activated test system. For cultures exposed for 4 hours, at 4 hours the treatment medium was removed, the cells were washed and re-fed with complete medium, and returned to the incubator for a further 16 hours. Two hours prior to harvest, Colcemid® was added to the cultures to arrest cells in metaphase. At 20 hours, cells were collected and processed to slides.

No evidence of test article precipitation or cytotoxicity was observed in the preliminary toxicity test. The dose levels selected for the definitive assay were 1000, 2500, 3500 and 5000 μg/mL for activated and non-activated 4 hour exposures and non-activated 20 hour exposure in the definitive study. The method of the definitive study was the same as that used in the preliminary study, although all cultures were conducted in duplicate. Slides from the 1000, 2500 and 5000 μg/mL groups in the definitive study were scored for metaphase chromosome aberrations in at least 200 metaphase spreads. The percentage of structurally aberrant cells was not significantly increased over that in negative control assays in the activated or non-activated 4 hour exposure groups, or in the 20 hour non-activated group. The percentage of structurally aberrant cells was significantly increased in all the positive control cultures, confirming the validity of the assay.

It was concluded that the test article was negative for induction of structural and numerical chromosome aberrations in both the non-activated and S9-activated test systems.

3.4.5 Potential for allergenicity

A full-length sequence alignment against known allergens in the Food Allergy Research and Resource Program (FARRP) AllergenOnline database, using an E-value <0.1, was conducted in March 2018 and yielded no matches. An 80 amino acid sliding window search of the same database, for ≥ 35% identity to known allergens, likewise revealed no matches.

The enzyme preparation contains wheat starch and wheat flour. Wheat bran, glucose from wheat, and soy flour, used in the fermentation process, may be present.

3.4.6 Approvals by other regulatory agencies

A letter of approval for the enzyme from the Ministry of Environment and Food in Denmark was provided by the applicant.

The US-FDA responded with a No Questions letter to a GRAS Notice (GRN 567) for xylanase from *T. reesei*, carrying the xylanase gene from *A. niger* (var. *tubingensis*), in 2015. According to the applicant, that xylanase is similar to the enzyme that is the subject of this application.

4   Discussion

There are no public health or safety concerns for the general population associated with the use of this endo-1,4-beta-xylanase as a food processing aid.

This enzyme has a history of safe use in other countries, including Norway and Peru, in use in these countries since 2016. In addition, FSANZ has already approved the endo-1,4-beta-xylanase synthesized by the donor organism, *A. niger*. The current enzyme is produced by *T. reesei* genetically modified to synthesize the endo-1,4-beta-xylanase of *A. niger*.

Molecular evidence confirmed the taxonomy of the production strain of *T. reesei* containing the endo-1,4-beta-xylanase gene. This fungus is not toxigenic or pathogenic, and has a long history of safe use in the production of a number of enzyme processing aids that are already permitted in the Code. Analysis of the production strain confirmed the presence of the inserted gene.

The enzyme showed no evidence of genotoxicity in a bacterial reverse mutation assay or a chromosomal aberration assay. In a 90-day oral gavage study in rats, the NOAEL was the highest dose tested, 1000 mg/kg bw/day total protein, which is equivalent to 1214 mg/kg bw/day TOS. The TMDI was calculated by the Applicant to be 0.635 mg/kg bw/day TOS[4]. Comparison of the NOAEL and the TMDI gives a Margin of Exposure (MoE) of approximately 1900.

Bioinformatic data indicated a lack of homology with known toxins or allergens. The enzyme preparation contains wheat starch and wheat flour, while wheat bran, glucose from wheat, and soy flour may be present.

5   Conclusions

Based on the reviewed data it is concluded that in the absence of any identifiable hazard an Acceptable Daily Intake (ADI) 'not specified' is appropriate. A dietary exposure assessment was therefore not required.

6   References

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[4] The original application quoted a TMDI of 0.488 mg/kg bw/day as reported in the Call for Submissions SD1. The applicant subsequently provided a revised TMDI of 0.635 mg/kg bw/day.

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