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Supporting document

Risk and Technical Assessment – Application A1267

Fructanase from GM *Trichoderma reesei* as a processing aid

Executive summary

AB Enzymes GmbH (AB Enzymes) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme fructanase (EC 3.2.1.80) as a processing aid in the manufacture of bakery products such as bread, steamed bread, bread buns, tortillas, cakes, pancakes and waffles. The enzyme is a protein engineered variant of fructanase from *Lactobacillus crispatus*, produced by genetically modified (GM) *Trichoderma reesei*.

The proposed use of the fructanase enzyme as a processing aid in the manufacture of bakery products is consistent with its typical function of catalysing the hydrolysis of fructans. Fructanase performs its technological purpose during the production of food and is not performing the technological purpose in the food for sale. It is therefore functioning as a processing aid for the purposes of the Code.

There are relevant identity and purity specifications for the enzyme in the Code and the applicant provided evidence that their enzyme meets these specifications.

T. reesei has a long history of safe use as a production microorganism of enzyme processing aids, including several that are already permitted in the Code. The production organism is neither pathogenic nor toxigenic. Analysis of the genetically modified production strain confirmed the presence and stability of the inserted DNA.

Bioinformatics analysis found no significant homology of the fructanase enzyme itself with any known toxins or food allergens. Studies with another enzyme, cellulase, from a production strain within the same safe strain lineage as the strain used to produce fructanase found no evidence of genotoxicity and no adverse effects in a 90-day oral toxicity study in rats, confirming the safety of the production strain. The NOAEL in this study was 1000 mg total organic solids per kilogram body weight per day (TOS/kg bw/day), the highest dose tested.

The theoretical maximum daily intake (TMDI) of the TOS from the fructanase enzyme preparation was calculated to be 0.21 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI results in a large Margin of Exposure (MOE) of approximately 4,800.

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.

Wheat flour is used as an ingredient in the enzyme preparation.

Contents

1 Introduction

AB Enzymes GmbH (AB Enzymes) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of a protein engineered variant of the enzyme fructanase (EC 3.2.1.80) as a processing aid in the manufacture of bakery products such as bread, steamed bread, bread buns, tortillas, cakes, pancakes and waffles. This fructanase is produced from a genetically modified (GM) strain of *Trichoderma reesei*., containing the fructanase gene from *Lactobacillus crispatus.*

The objectives of this technical and risk assessment were to:

- determine whether the proposed purpose is a solely technological purpose and that the enzyme achieves its technological purpose as a processing aid in the quantity and form proposed to be used
- evaluate potential public health and safety issues that may arise from the use of this enzyme, as a processing aid in the manufacture of bakery products, specifically by considering the:
  - safety and history of use of the host and gene donor organisms
  - characterisation of the genetic modification(s), and
  - safety of the enzyme.

2 Food technology assessment

2.1 Identity of the enzyme

The applicant provided relevant information regarding the identity of the fructanase enzyme. FSANZ verified this using the IUBMB[1] enzyme nomenclature database (McDonald et al 2009) and the enzyme database BRENDA[2] (Chang et al 2021). Details of the identity of the enzyme are provided below.

Accepted IUBMB name:	fructan β-fructosidase
Systematic name:	β-D-fructan fructohydrolase
Other names:	exo-β-D-fructosidase; exo-β-fructosidase; polysaccharide β-fructofuranosidase; fructan exohydrolase, fructanase
EC number:	3.2.1.80
Reactions:	Hydrolysis of terminal, non-reducing (2→1)- and (2→6)-linked β-D-fructofuranose residues in fructans

The hydrolysis reaction scheme for fructanase is available under its record in the enzyme database BRENDA (Chang et al 2021).

[1] International Union of Biochemistry and Molecular Biology. EC 3.2.1.80 (qmul.ac.uk)
[2] Information on EC 3.2.1.80 - fructan beta-fructosidase - BRENDA Enzyme Database (brenda-enzymes.org)

2.2 Manufacturing process

2.2.1 Production of the enzyme

The fructanase that is the subject of this application is produced by submerged fermentation of a genetically modified strain of *T. reesei* containing the fructanase gene from *L. crispatus.* The applicant notes that the fermentation process used is substantially equivalent across the world. The fermentation steps are inoculum, seed fermentation and main fermentation. This is followed by the recovery stage which involves primary solid/liquid separation, concentration to achieve the desired enzyme activity and/or to increase the ratio of enzyme activity to total organic solids (TOS) before formulation, then polish and germ filtration, to provide a concentrated enzyme solution free of the production strain and insoluble substances.

This is followed by formulation of the enzyme into an enzyme preparation. Enzymes are generally sold as enzyme preparations, which consist of the enzyme(s) and other ingredients to facilitate their storage, sale, standardisation, dilution or dissolution. The applicant's fructanase enzyme preparation is mainly sold as a powdered product. The typical composition of their enzyme preparation is:

Fructanase enzyme concentrate	2-4%
Sunflower oil	0.2%
Wheat flour	remainder

The application states that the enzyme is produced in accordance with current Good Manufacturing Practice for Food (cGMP) and the principles of Hazard Analysis and Critical Control Point (HACCP). It also states that all raw materials used in the fermentation and recovery processes are standard ingredients of food grade quality that meet predefined quality standards. The raw materials conform to either specifications set out in the Food Chemicals Codex, 13th edition, 2022 or regulations applying in the European Union.

Details on the manufacturing process, raw materials and ingredients used in the production of the fructanase enzyme preparation were provided in the application, some as Confidential Commercial Information (CCI).

2.2.2 Specifications for identity and purity

There are international general specifications for enzyme preparations used in the production of food. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its Compendium of Food Additive Specifications (FAO/WHO 2006) and in the Food Chemicals Codex (FCC 2020). These specifications are included in earlier publications of the primary sources listed in section S3—2 of Schedule 3 of the Code, and enzymes used as a processing aid must meet either of these specifications. In addition, under JECFA, enzyme preparations must meet the specifications criteria contained in the individual monographs. In the case of fructanase, there is no individual monograph.[3]

Schedule 3 of the Code also includes specifications for arsenic and heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

The applicant provided the results of analysis of four different batches of their fructanase enzyme concentrate. Table 1 provides a comparison of the analyses with international specifications established by JECFA and the FCC, as well as those in the Code (as

[3] For the functional use 'enzyme preparation', the JECFA database can be searched for individual monographs: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/

applicable). Based on these results, the enzyme met the relevant specifications in Schedule 3 of the Code.

Table 1 *Comparison of the applicant's fructanase enzyme concentrate compared to JECFA, Food Chemicals Codex and the Code specifications for enzymes*

Analysis	Results from applicant	JECFA	Food Chemicals Codex	Australia New Zealand Food Standards Code (section S3-4)
Lead (mg/kg)	<0.05	≤5	≤ 5	≤2
Arsenic (mg/kg)	<0.5	-	-	≤1
Cadmium (mg/kg)	<0.03	-	-	≤1
Mercury (mg/kg)	<0.05	-	-	≤1
Coliforms (cfu/g)	<30	≤30	≤30	-
***Salmonella* (in 25 g)**	Not detected	Absent	Negative	-
***E. coli* (in 25 g)**	Not detected	Absent	-	-
Antimicrobial activity	Not detected	Absent	-	-

The applicant stated that the absence of the production strain is confirmed by an in-house method for every batch of the enzyme preparation.

2.3 Technological purpose and justification

The applicant requested permission to use the enzyme fructanase as a processing aid in the manufacture of bakery products such as bread, steamed bread, bread buns, tortillas, cakes, pancakes and waffles. The enzyme would be used at a level not higher than necessary to achieve the desired enzyme reaction under Good Manufacturing Practice (GMP).

Fructanase is a glycosidase, belonging to the hydrolase enzyme class. It catalyses hydrolysis of terminal, non-reducing (2→1)- and (2→6)-linked beta-D-fructofuranose residues in fructans. Fructans are polymers of fructose with the fructose units primarily joined by two types of molecular linkages, inulin type beta (2→1) and levan type beta (2→6) (Carabin et al 1999).

Fructanase hydrolyses inulin, levan and sucrose (IUBMB 2018). These substrates are commonly found in plants including cereal grains such as wheat and cereal grain products such as wheat flour used in baking.

The applicant refers to the substrates for fructanase as 'fructo-oligosaccharide (FOS) and related polysaccharides'. FOS consist of a chain of fructose units with a terminal glucose unit linked by beta (2→1) glycosidic bonds (Sabater-Molina et al, 2009). The reference to 'FOS and related polysaccharides' by the applicant is consistent with the reference to fructans outlined above.

The applicant stated that FOS and related polysaccharides cause technical difficulties due to viscosity in the processing of raw materials containing those components. They stated that the use of fructanase assists in food processing, including by reducing viscosity and improving processability, enhancing yields and shortening processing times, leading to better or more consistent product characteristics and helping to achieve more effective production processes.

The applicant provided papers from the scientific literature to support these technological functions. Donatella et al (2009) and Sirbu and Arghire (2017) found that the addition of inulin to wheat dough resulted in an increase in mixing time and a reduction in water absorption. The applicant noted that a reduction in inulin by hydrolysis by fructanase is therefore likely to have the opposite effect. The degradation of fructans by fructanase also increased the level of fermentable and reducing sugars in dough (Loponen and Gänzle, 2018).

The applicant's fructanase enzyme preparation is a light beige powder. The applicant reports that the enzyme is denatured by heat at temperatures above 80°C. The applicant provided additional information on the chemical properties of the enzyme preparation as CCI. Based on the information provided by the applicant, FSANZ agrees that the enzyme performs its primary technological functions during food processing of the nominated foods (bakery products). It will be denatured during the baking process and have no technological effect in the final food. As such, the enzyme functions as a processing aid for the purposes of the Code.

2.4 Food technology conclusion

FSANZ concludes that the proposed use of this fructanase enzyme as a processing aid in the manufacture of bakery products is consistent with its typical function of catalysing the hydrolysis of fructans.

Fructanase performs its technological purpose during the production of food and is not performing the technological purpose in the food for sale. It is therefore functioning as a processing aid for the purposes of the Code.

There are relevant identity and purity specifications for the enzyme in the Code and the applicant provided evidence that their enzyme meets these specifications.

3 Safety assessment

The objective of this safety assessment was to evaluate any potential public health and safety concerns that may arise from the use of this enzyme as a processing aid.

Some information relevant to this section is CCI, so full details cannot be provided in this public report.

3.1 History of use

3.1.1 Host organism

Trichoderma reesei

FSANZ has previously assessed the safety of *T. reesei* as the source organism for at least 15 processing aids in Schedule 18. Several enzymes produced by *T. reesei* QM6a have Generally Recognized as Safe (GRAS) status with the Food and Drug Administration (FDA)

or FDA had no questions about the GRAS conclusions about them contained in GRAS submissions to FDA (US EPA 2012).

There is a long history of safe industrial use of *T. reesei* as a safe production microorganism to produce enzymes for food as well as feed processing and numerous other industrial applications. *Trichoderma reesei* is a common, hypercellulolytic, soil fungus that was initially isolated from deteriorating canvas made from cellulosic material. The original isolate QM6a is the type strain for *T. reesei* (Olempska-Beer et al*.,* 2006) and has been registered with the American Type Culture Collection under ATCC13631. Strain QM6a is the wild type of practically all *T. reesei* industrial production strains (Nevalainenet et al., 1994). The production strain *T. reesei* AR-577 has been derived from a safe strain lineage, including strain AR-852, originally deriving from *T. reesei* QM6a. The data provided in the application shows that *T. reesei* AR-577 is safe to use as the production organism for fructanase enzyme.

T. reesei QM6a strains are non-pathogenic, not known to possess any virulence factors associated with colonisation or disease, and do not present any human toxicity concerns (US EPA 2012). Several review papers support the safety of *T. reesei* QM6a strains with no production of known mycotoxins or antibiotics under conditions used for enzyme production (Nevalainen et al., 1994; Hjortkjaer et al. 1986; Coenen et al., 1995; Cardoza et al., 2011; JECFA, 2006; Hjortkjaer et al., 1986; Kubicek et al., 2007; Parekh et al., 2000; Sanchez and Demain, 2002). *T. reesei* QM6a strains are known to produce peptaibol antibiotic paracelsin, but industry-standard submerged fermentation conditions are not linked to the production of paracelsin (US EPA 2012).

T. reesei can be used under the lowest containment level at large scale, GILSP (Good Industrial Large Scale Practice), as defined by OECD (Organisation for Economic Co-operation and Development (OECD, 1992). *T. reesei* is listed as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BAuA, 2016) and meets the requirements of a Biosafety Level 1 organism based on the Biosafety in Microbiological and Biomedical Laboratories guidelines[4].

Data provided with the application confirmed the identity of the production strain as *T. reesei*. The analysis of characteristics of four representative batches of enzyme and production methodology demonstrated that culture conditions can be applied consistently between batches. Results confirming that viable cells of the production organism are not detected in the final enzyme production were provided. There was no antimicrobial activity of the enzyme preparation following analysis according to JECFA methods (JECFA, 2006).

[4] https://www.cdc.gov/biosafety/publications/bmbl5/index.htm

3.1.2 Gene donor organism

Lactobacillus crispatus

The donor organism of the fructanase gene is *Lactobacillus crispatus* and meets the requirements of a Biosafety Level 1 organism.

3.2 Characterisation of the genetic modification(s)

3.2.1 Description of the DNA to be introduced and method of transformation

An expression cassette containing a gene encoding a protein engineered variant of the fructanase enzyme was introduced into the genome of the host *T. reesei* strain using standard methodologies. The fructanase gene is from *Lactobacillus crispatus* and was placed under the control of a *T. reesei* promoter and terminator. The expression cassette also contained the *amdS* selectable marker gene from *Aspergillus nidulans*, allowing transformants to be selected based on their ability to grow on media supplemented with acetamidase. Data provided by AB enzymes and analysed by FSANZ confirmed the identity of the fructanase enzyme. The enzyme has been protein engineered.

3.2.2 Characterisation of inserted DNA

Southern blot data provided by AB Enzymes and analysed by FSANZ confirmed the presence of the inserted DNA in the production strain. The applicant also provided the results of whole genome sequencing which confirmed the absence of antibiotic resistance genes in the production strain.

3.2.3 Genetic stability of the inserted gene

The assessment confirmed that the inserted gene is integrated into the genome of the production strain and does not have the ability to replicate autonomously. The inserted gene is therefore considered to be genetically stable.

To provide further evidence of the stability of the introduced gene, the applicant provided phenotypic data from large-scale fermentation of the production strain. These data confirmed that the gene encoding the fructanase enzyme is expressed over multiple generations and is stable.

3.3 Safety of the fructanase enzyme

3.3.1 History of safe use

No fructanase enzymes are currently permitted in the Code and fructanase from GM *T. reesei* does not have a documented history of safe use.

3.3.2 Bioinformatic assessment of toxicity

The applicant performed a BLAST-P search for homology of the amino acid sequence of the enzyme and that of known protein toxins, performed in September 2022. No significant homology was identified.

3.3.3 Toxicity studies

The applicant submitted toxicity studies performed with an enzyme (cellulase) produced by a *T. reesei* strain (AR-852) in the same safe strain lineage as the production strain for

fructanase (*T. reesei* AR-577). *T. reesei* AR-852 is derived from the same intermediate strain as *T. reesei* AR-577.

FSANZ compared the enzyme specifications supplied by the applicant, reviewed the genetic differences between the two production strains and compared the manufacturing processes. Based on this evaluation, the test item used in the toxicity studies is considered suitably equivalent for assessing the safety of *T. reesei* AR-577 and the fructanase enzyme.

Animal studies

90-day oral toxicity study in rats ([Redacted] 2020) Regulatory status: GLP; conducted in accordance with OECD test guideline (TG) 408

Cellulase was administered to Wistar rats (10/sex/group) at doses of 0, 100, 300 or 1000 mg total organic solids (TOS)/kg bw/day for 90 days. Water was used as the vehicle control. Animals were monitored daily for clinical signs of toxicity. Body weight and food consumption were recorded weekly. Ophthalmological examinations were performed on all animals before the study and in the final week of treatment. Detailed behavioural observations were made using a functional observation battery of tests prior to treatment and during week 11. At the end of the study blood and urine samples were collected for haematology, coagulation, clinical biochemistry and thyroid hormone analyses. Animals were subjected to gross necropsy and organ weight analysis. Histopathological examination was performed on organs and tissues from the control and high dose groups, as well as any gross macroscopic lesions.

All animals survived to the end of the study and no treatment-related clinical signs were observed. There were no treatment-related adverse effects on ophthalmology, functional observation battery, haematology, coagulation, clinical chemistry and thyroid hormone analyses. No treatment-related changes in gross findings, organ weights or histopathological examinations were observed.

The no observed adverse effect level (NOAEL) was 1000 mg TOS/kg bw/day, the highest dose tested.

Genotoxicity studies

Bacterial reverse mutation test ([Redacted] 2019) Regulatory status: GLP; conducted in accordance with OECD TG 471

The potential mutagenicity of cellulase was evaluated in *Salmonella enterica* ser. Typhimurium strains TA98, TA100, TA1535, TA1537 and TA102, with and without metabolic activation using rat liver homogenate (S9). The maximum dose and dose range evaluated was consistent with the OECD TG recommendations. Two independent experiments were performed, one using the plate incorporation method and one with the preincubation method. Positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535), 4-nitro-o-phenylene (TA98 and TA1537) and methylmethane sulphonate (MMS; TA102). The positive control in the presence of metabolic activation was 2-aminoanthracene (all strains). Water was used as the vehicle control.

No concentration-related increases in revertant colonies were observed in cultures treated with the test item, relative to vehicle controls, with or without metabolic activation. All positive control treatments showed the anticipated increases in mutagenic activity demonstrating the validity of the assay.

It was concluded that the cellulase test item was not mutagenic under the conditions of this

test.

In vitro mammalian cell micronucleus test in human lymphocytes ([Redacted] 2019)
Regulatory status: GLP; conducted in accordance with OECD TG 487

The potential of cellulase to induce micronuclei was tested using human peripheral blood lymphocytes. Treatment with the test item was either a 4 hour exposure in the absence or presence of metabolic activation (S9) followed by culture in medium containing cytochalasin B for 40 hours, or 44 hours continuous exposure without S9 in the presence of cytochalasin B. RPMI culture medium was used as the vehicle control. Clastogenic positive controls were MMS in the absence of S9 and cyclophosphamide in the presence of S9. Colchicine was used as the aneugenic positive control.

As a result of dose selection experiments and cytotoxicity observations, concentrations evaluated for micronucleus frequencies were 250 – 2500 µg/mL and 125 – 200 µg/mL in the 4-hour and 44-hour treatments without S9, respectively. Concentrations of 500 – 4000 µg/mL were evaluated in the 4-hour treatment with S9.

There were no significant increases in the incidence of micronucleated human lymphocytes following exposure the test item relative to the vehicle controls under any of the test conditions. The positive controls demonstrated a statistically significant increase in micronuclei formation, confirming the validity of the assay.

It was concluded that cellulase was not clastogenic or aneugenic under the conditions of this study.

3.3.4 Potential for allergenicity

Searches for homology of the fructanase amino acid sequence with those of known allergens were performed in 2022 using the AllergenOnline database[5]. Three searches were performed:

- Alignment (FASTA) of the entire query amino acid sequence to known allergens (more than 35% identity)
- Alignment (FASTA) of sliding 80-amino acid windows of the query protein to known protein allergens (more than 35% identity)
- A search for 8 amino acid exact matches.

No matches of greater than 35% identity were found using the full-length search or the 80-mer sliding window search. No exact matches of 8 amino acids were found.

Wheat flour is used as an ingredient in the enzyme preparation.

3.4 Dietary exposure assessment

The objective of the dietary exposure assessment was to review the budget method calculation presented by the applicant as a ‘worse-case scenario’ approach to estimating likely levels of dietary exposure, assuming that all of the TOS from the fructanase enzyme preparation remained in the food.

The budget method is a valid screening tool for estimating the theoretical maximum daily intake (TMDI) of a food additive (Douglass et al., 1997). The calculation is based on physiological food and liquid requirements, the food additive concentration in foods and

[5] AllergenOnline: http://www.allergenonline.org/

beverages, and the proportion of foods and beverages that may contain the food additive. The TMDI can then be compared to an ADI or a NOAEL to estimate a margin of exposure for risk characterisation purposes. Whilst the budget method was originally developed for use in assessing food additives, it is also appropriate to use for estimating the TMDI for processing aids (FAO/WHO, 2020). The method is used by international regulatory bodies and the FAO/WHO Joint Expert Committee on Food Additives (JECFA) (FAO/WHO, 2021) for dietary exposure assessments for processing aids.

In their budget method calculation, the applicant made the following assumptions:

- the maximum physiological requirement for solid food (including milk) is 25 g/kg body weight/day
- 50% of solid food is processed
- all solid processed foods contain the highest use level of 12 mg TOS/kg in the raw material (flour)
- a ratio 0.71 for raw material (flour) weight to final food (bakery products) weight
- the maximum physiological requirement for liquid is 100 mL/kg body weight/day (the standard level used in a budget method calculation for non-milk beverages)
- 25% of non-milk beverages are soft drinks
- the enzyme preparation is not added to any non-milk beverages
- all of the TOS from the enzyme preparation remains in the final food.

Based on these assumptions, the applicant calculated the TMDI of the TOS from the enzyme preparation to be 0.107 mg TOS/kg body weight/day.

As assumptions made by the applicant differ from those that FSANZ would have made in applying the budget method, FSANZ independently calculated the TMDI using the following assumptions that are conservative and reflective of a first tier in estimating dietary exposure:

- The maximum physiological requirement for solid food (including milk) is 50 g/kg body weight/day (the standard level used in a budget method calculation where there is potential for the enzyme preparation to be in baby foods or general purpose foods that would be consumed by infants).

- FSANZ would generally assume 12.5% of solid foods contain the enzyme preparation based on commonly used default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO, 2009). However, the applicant has assumed a higher proportion of 50% based on the nature and extent of use of the enzyme and therefore FSANZ has also used this proportion for solid foods as a worst case scenario.

All other inputs and assumptions used by FSANZ remained as per those used by the applicant. The TMDI of the TOS from the enzyme preparation based on FSANZ's calculations is 0.21 mg TOS/kg body weight/day.

Both the FSANZ and applicant's estimates of the TMDI will be overestimates of the dietary exposure given the conservatisms in the budget method. This includes that it was assumed that all of the TOS from the enzyme preparation remains present and active in the final foods whereas the applicant has stated that the enzyme would be inactivated by heat during processing (baking) and does not perform any technological function in the final food.

4 Discussion

No public health and safety concerns were identified in the assessment of fructanase

produced by GM *T. reesei*. *T. reesei* has a long history of safe use as a source of enzyme processing aids, including several that are already permitted in the Code. The *T. reesei* host is neither pathogenic or toxigenic. Analysis of the GM production strain confirmed the presence and stability of the inserted DNA.

Bioinformatics analysis found no significant homology of the fructanase enzyme itself with any known toxins or food allergens. Studies with another enzyme, cellulase, from a production strain within the same safe strain lineage as the strain used to produce fructanase found no evidence of genotoxicity and no adverse effects in a 90-day oral toxicity study in rats, confirming the safety of the production strain. The NOAEL in this study was 1000 mg TOS/kg bw/day, the highest dose tested.

The TMDI of the TOS from the fructanase enzyme preparation was calculated to be 0.21 mg TOS/kg bw. A comparison of the NOAEL and the TMDI results in a Margin of Exposure (MOE) of approximately 4,800.

Wheat flour is used as an ingredient in the enzyme preparation.

5 Conclusion

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.

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