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SCIENTIFIC OPINION

Scientific Opinion on thrombin from cattle (bovines) and pig's blood[1]

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)[2,3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The food enzyme considered in this opinion is thrombin (EC 3.4.21.5) obtained from blood plasma of cattle and pigs. It is obtained from cattle and pig's blood that is fit for human consumption and is processed hygienically, without artificial changes. As the food enzyme is derived from edible parts of animals, no toxicological tests are required. The dietary exposure was assessed in accordance with the EFSA Comprehensive European Food Consumption Database. Based on the origin of the food enzyme from edible parts of animals, the manufacturing process, and the compositional and biochemical data provided, the Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use.

KEY WORDS

food enzyme, thrombin, fibrinogen, cattle blood, pig's blood, meat

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SUMMARY

Following a request from the European Commission, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF Panel) was asked to deliver a scientific opinion on the food enzyme thrombin (EC 3.4.21.5) obtained from blood plasma of cattle and pigs.

The food enzyme is obtained from cattle and pig's blood that is fit for human consumption and is processed hygienically. The food enzyme is used together with added fibrinogen; the thrombin catalyses the transformation of fibrinogen to fibrin, which interacts with collagen, enabling the binding of meat/fish pieces resulting in meat preparations, meat products and fishery products.

The typical use and the recommended maximum use levels of the food enzyme have been provided.

Dietary exposure to thrombin from its use as a food enzyme was estimated using the EFSA Comprehensive European Food Consumption Database. The estimated mean and 95th percentile exposure across five population groups ranged from 3 to 12 µg TOS/kg body weight/day and from 6 to 24 µg TOS /kg bw/day, respectively.

The food enzyme has been characterised by determining the temperature and pH optima and the thermostability. Its composition is characterised by measuring the enzyme activity, content of protein and total organic solids.

As the food enzyme is derived from edible parts of animals, no toxicological tests are required.

Considering the origin of the food enzyme, the CEF Panel considers that the likelihood of a food allergic reaction to this thrombin is low and, therefore, it does not give rise to safety concerns.

Based on the origin of the food enzyme from edible parts of animals, the manufacturing process, and the compositional and biochemical data provided, the Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use.

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1. Introduction

1.1. Background and Terms of Reference as provided by European Commission

Only food enzymes included in the Union list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7 (2) of Regulation (EC) No 1332/2008[4] on food enzymes. According to Regulation (EC) No 1332/2008 on food enzymes, a food enzyme which falls within the scope of Regulation (EC) No 1829/2003[5] on genetically modified food and feed should be authorised in accordance with that Regulation as well as under this Regulation.

An application has been introduced by the company Sonac for the authorisation of the food enzyme thrombin obtained from blood plasma of cattle and pigs.

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011[6] implementing Regulation (EC) No 1331/2008[7], the Commission has verified that the application falls within the scope of the food enzyme Regulation and contains all the elements required under Chapter II of that Regulation.

The European Commission requests the European Food Safety Authority to carry out the safety assessment on the food enzyme thrombin obtained from blood plasma of cattle and pigs in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

2. Assessment

2.1. Introduction

Before January 2009, food enzymes other than those used as food additives were not regulated or were regulated as processing aids under the legislation of the Member States. On 20 January 2009, Regulation (EC) No 1332/2008 on food enzymes entered into force. This regulation applies to enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. Regulation (EC) No 1331/2008 established European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

- it does not pose a safety concern to the health of the consumer at the level of use proposed;
- there is a reasonable technological need; and
- its use does not mislead the consumer.

All food enzymes currently on the EU market and intended to remain on the market, as well as all new food enzymes, shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and an approval via an EU list.

[4] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/199, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EU) No 258/97. OJ L 354, 31.12.2008, p. 7–15.

[5] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003 p. 1–23.

[6] Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.3.2011, p. 15–24.

[7] Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, p. 1–6.

The Guidance on submission of a dossier on a food enzyme for evaluation by EFSA (EFSA CEF Panel, 2013) lays down the administrative, technical and toxicological data required.

The following evaluation applies to the thrombin obtained from blood plasma of cattle and pigs.

2.2. Information on existing authorisations and evaluations

The applicant reports that EFSA evaluated an enzyme preparation based on thrombin: fibrinogen with the outcome of no safety concern (EFSA CEF Panel, 2005). The use of thrombin has been approved in the Netherlands, the USA, Australia and New Zealand.

2.3. Technical data

2.3.1. Identity of the food enzyme

International Union of Biochemistry and Molecular Biology (IUBMB) nomenclature: thrombin.

Synonyms: fibrinogenase; thrombase; thrombofort; topical; thrombin-C; tropostasin; activated blood-coagulation factor II; blood-coagulation factor IIa; factor IIa; E thrombin; β-thrombin; γ-thrombin.

IUBMB No: EC 3.4.21.5.

Chemical Abstracts Service (CAS) No: 9002-04-4.

European Inventory of Existing Commercial chemical Substances (EINECS) No: 232-648-7.

2.3.2. Chemical parameters

Thrombin is a serine protease involved in the final step of blood coagulation. It is isolated from blood plasma from cattle (bovines) and pigs. Thrombin consists of two polypeptide chains with an apparent molecular mass of about 36.5 kDa and has an isoelectric point of 7.5. The proteins all originate from the original blood plasma. The enzyme is not modified by technological procedures or other means, and is not subject to protein engineering.

Thrombin is diluted in water with calcium chloride, without any preservatives. The solution is stored below –18 °C. The average total organic solids (TOS) of this solution is 0.02 % and the average activity of the thrombin enzyme in this solution is 20.54 National Institute of Health (NIH) units per ml. Accordingly, the ratio of enzyme activity/mg to TOS is 102.7 NIH units per mg TOS (Table 1).

Table 1: Compositional data of the food enzyme

Parameter	Unit	Values
Enzyme activity [(a)]	NIH/ml	20.54 ± 1.04
Protein	g/kg	0.2
TOS	%	0.02
Activity/mg TOS	NIH/mg TOS	102.7
Ca^{2+}	g/kg	22.0
Na^{+}	g/kg	2.0

(a): The enzyme activity is the mean of 182 batches.
The corrected standard deviation from the measured NIH/ml is 0.74 NIH units based on 74 measurements over time in one batch.

The microbiological criteria provided by the applicant for the food enzyme are as follows:

- Total bacterial count < 100 000 CFU (colony-forming units)/g
- *Salmonella* spp. negative/25 g
- *Staphylococcus aureus* < 1 000 CFU/g
- Enterobacteriaceae < 1 000 CFU/g
- Moulds < 100 CFU/g
- Yeasts < 100 CFU/g
- *Bacillus* < 100 CFU/g
- *E. coli* < 100 CFU/g
- *Clostridium* spp. < 100 CFU/g
- Coliforms < 100 CFU/g
- *Streptococcus* spp. < 1 000 CFU/g.

2.3.3. Properties of the food enzyme

Thrombin has a specific proteolytic activity, and cleaves certain arginine–glycine peptide bonds in fibrinogen (Berg et al., 2012). The thrombin is added to meat, together with concentrated fibrinogen. Fibrinopeptides A and B are split from fibrinogen, after which the fibrin molecules form fibrin fibres.

The activity of thrombin is measured with a chromogenic substrate at pH 8.0 and 37 °C. Thrombin cleaves the substrate and releases *p*-nitroaniline (*p*NA). The thrombin activity is expressed as NIH units. The amount of *p*NA generated, measured in a spectrophotometer at 405 nm, is directly proportional to thrombin activity. The optimal pH for thrombin is pH 8 in the presence of at least 0.1 M sodium chloride (Orthner and Kosow, 1980; Barrett et al., 2004).

The stability of thrombin is rather low. Its activity decreases very rapidly with increased temperature, having a half-life of 60 and 6 minutes when incubated at 25 and 45 °C, respectively (Le Borgne and Graber, 1994). Once the binding process is achieved (overnight), no residual thrombin activity can be detected at a detection limit of 0.004 NIH units per ml (EFSA CEF Panel, 2005).

2.3.4. Information on the animal source materials

According to the applicant, thrombin is isolated from blood plasma of healthy slaughtered porcine (*Sus scrofa domesticus*) or bovine (*Bos taurus*) species that are fit for human consumption.

Blood is hygienically collected in approved EU slaughterhouses. Coagulation of blood is prevented by the addition of citrate. The blood is then treated in accordance with good hygienic practices and cooled back to 3 °C or below. To ensure the safety of the blood and the produced thrombin and fibrinogen thereof, the slaughterhouses and blood treatment plants operate a hazard analysis and critical control points (HACCP) system to control the relevant risks.

The applicant has provided information to ensure the absence of any risk of infectivity (including transmissible spongiform encephalopathies (TSEs).

2.3.5. Manufacturing process

The manufacturing process for thrombin starts with the hygienic collection of blood at the slaughterhouse, after stunning of the animals, from only those animals fit for human consumption. Blood coagulation is inhibited by the addition of citric acid to the blood (average 0.7–0.8 %). The blood is stored in tanks and cooled at 3 °C until reaching the production plant.

Blood cells and plasma are separated by centrifugation. The plasma is further separated by pure physical means into fibrinogen-enriched plasma and defibrinated plasma. Prothrombin is separated from the defibrinated plasma by ion-exchange chromatography. Prothrombin, diluted in water, is activated to thrombin by meat thromboplastin from a meat extract in the presence of a calcium chloride solution. The meat extract is made from shoulder muscles of beef or pork fit for human consumption that are homogenised in a sodium phosphate solution and centrifuged. The supernatant is collected and used as a meat extract. After activation, the thrombin solution is reported by the applicant to contain approximately 20 NIH units per ml of thrombin (Table 1), sodium chloride, sodium citrate and calcium chloride. The food enzyme is frozen and stored below –18 °C.

According to the petitioner, thrombin is produced under hygienic conditions in accordance with Regulation (EC) No 853/2004 to process meat. Prerequisite requirements and an operational HACCP system are implemented. Specifically, the temperature of the blood at collection is checked and the blood is stored under cooled conditions in accordance with Regulation (EC) No 853/2004. The blood in the storage tank is continuously monitored. After production, the food enzyme is stored below –18 °C. The temperature in the storage room is continuously measured by an automated system with alarms. Every batch is microbiologically and biochemically tested before release.

2.3.6. Reaction and fate in food

Thrombin catalyses the transformation from fibrinogen to fibrin; fibrin fibres then interact with collagen, enabling the binding of meat pieces and resulting in a formed meat product; the same applies to fish. After six hours, the final strength of the binding is reached. In that time, the fibrin strings are cross-linked like in the normal coagulation process. Calcium is an important co-factor in the coagulation process. The process temperature is below 7 °C. After fibrin formation, the remaining thrombin will be inactivated by anti-thrombin (anti-thrombin III) and by binding to the formed fibrin (Rau et al., 2007).

In addition to the added thrombin, there is intrinsic thrombin activity in meat and, to a lower extent, in fish also. After the formation process, the remaining thrombin activity is lower than or equal to the intrinsic thrombin activity in fresh non-treated meat. The typical content of prothrombin in meat is about 0.1–0.9 mg per kg meat, originating from the remaining blood (Warriss, 1984). This means that up to 0.4 mg thrombin per kg meat can be formed from the blood that remains in meat after slaughter.

Regarding subsidiary/side effects, the biologically normal reactions from the coagulation cascade occur when thrombin and fibrinogen are added to the meat. All the coagulation factors are present in the fibrinogen-enriched plasma, as are the factors that finally limit the coagulation, such as the anti-thrombin activity and binding of thrombin to fibrinogen.

2.3.7. Case of need and intended conditions of use

Thrombin is intended to be used in meat preparations, meat products and fishery products. Thrombin has to work together with added fibrinogen. Typical uses provided by the applicant are listed in Table 2.

Table 2: Typical uses and recommended maximum use levels of the food enzyme as provided by the applicant

Process	Recommended dosage of the food enzyme
Binding of meat or fish pieces in order to obtain meat preparations, meat products and fishery products	Up to 200 thrombin NIH units per kg food, corresponding to 1.95 mg TOS/kg food

According to the applicant, there is a technological need to use thrombin in combination with fibrinogen-enriched plasma to form portion-controlled meat or fish parts. This is a sustainable way to use meat and fish resources for human consumption.

2.4. Dietary exposure

Since 2010, the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) has been populated with data from national information on food consumption at a detailed level. Competent authorities in European countries provide EFSA with data on the level of food consumption by the individual consumer from the most recent national dietary survey in their country (Guidance of EFSA "Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment" (EFSA, 2011a)).

The food consumption data gathered by EFSA were collected using different methodologies and thus direct country-to-country comparison should be made with caution.

To calculate chronic exposure, intake statistics have been calculated based on individual average consumption over the total survey period excluding surveys with only one day per subject. High-level consumption was calculated only for those foods and population groups where the sample size was sufficiently large to allow calculation of the 95th percentile (EFSA, 2011b). The Panel estimated chronic exposure for the following population groups: toddlers, children, adolescents, adults and the elderly. Calculations were performed using individual body weights. Thus, for the present assessment, food consumption data were available from 26 different dietary surveys carried out in 17 different European countries.

Consumption records were codified in accordance with the FoodEx classification system (EFSA, 2011a). Nomenclature from the FoodEx classification system has been linked to the Food Classification System as presented in Annex II of Regulation (EC) No 1333/2008, part D, to perform exposure estimates.

2.4.1. Exposure to thrombin from its use as a food enzyme

Exposure was calculated using the food additives intake model (FAIM), available on the EFSA website (http://www.efsa.europa.eu/en/topics/topic/additives.htm). In the absence of data at sufficient levels of detail to identify products potentially containing the food enzyme, it was assumed that all of the food groups in FAIM (Table 3) contain the enzyme.

Table 3: Food groups in FAIM assumed containing the food enzyme

8 – Meat	8.1 – Unprocessed meat
8 – Meat	8.2 – Processed meat
9 – Fish and fisheries products	9.1.1 – Unprocessed fish
9 – Fish and fisheries products	9.1.2 – Unprocessed crustaceans and molluscs
9 – Fish and fisheries products	9.2 – Processed fish and fishery products including molluscs and crustaceans

Using this conservative assumption, the highest exposure was 24 µg TOS/kg body weight (bw)/day in toddlers (see Table 4). Since this enzyme application is probably used in only a small proportion of all meat, meat products and fishery products on the market, this figure is likely to be a significant overestimate.

Table 4: Anticipated exposure of five population groups to thrombin (mg TOS/kg bw/day) from its use as a food enzyme

Population group	Exposure to thrombin (mg TOS/kg bw/day)			
	Range for mean across dietary surveys		Range for high level across dietary surveys	
	Minimum	Maximum	Minimum	Maximum
Toddlers	0.0053	0.0118	0.0122	0.0238
Children	0.0056	0.0123	0.0106	0.0229
Adolescents	0.0032	0.0085	0.0080	0.0146
Adults	0.0033	0.0075	0.0055	0.0118
The elderly	0.0030	0.0042	0.0059	0.0071

2.5. Toxicological data

Toxicological tests are not requested, as the thrombin and the concentrated plasma with fibrinogen are derived from edible parts of animals, namely blood plasma, which is intended to be or reasonably expected to be ingested by humans (Regulation (EU) No 234/2011).

2.6. Allergenicity

All constituents of the food enzyme are normal constituents of blood and plasma and are ingested by consumers of meat. Blood and plasma proteins are not known to be food allergens. Thus, the likelihood of a food allergic reaction to this thrombin is considered to be low and, therefore, it does not give rise to safety concerns.

2.7. Discussion

The food enzyme is obtained from cattle (bovine) and pig's blood that is fit for human consumption and is processed hygienically.

The information provided on manufacturing of the food enzyme is considered sufficient. The available compositional data demonstrate the activity of the food enzyme and its variability.

Toxicological tests are not requested according to Regulation (EU) No 234/2011 and the CEF Guidance document, as thrombin and the concentrated plasma with fibrinogen are derived from edible parts of animals, namely blood plasma, which is intended to be or reasonably expected to be ingested by humans.

Dietary exposure estimates were calculated by assuming that the food enzyme is used in all food categories concerned at its maximum recommended dosage and remains in the final food. Based on these assumptions and considering the total intake, the estimated dietary exposure to thrombin is estimated to be up to 0.012 mg TOS/kg bw/day for adults and up to 0.024 mg TOS/kg bw/day for toddlers, which indicates a very low exposure.

Based on the origin of the food enzyme, the CEF Panel considers that the likelihood of food allergic reaction to this food enzyme is low and, therefore, it does not give rise to safety concerns.

3. Conclusions

Based on the origin of the food enzyme from edible parts of animals, the manufacturing process, and the compositional and biochemical data provided, the food enzyme thrombin derived from cattle (bovine) and pig's blood does not give rise to safety concerns under the intended conditions of use.

DOCUMENTATION PROVIDED TO EFSA

1. Dossier "Thrombin from cattle or pig blood". Submitted by Sonac, June 2014.

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ABBREVIATIONS

bw	body weight
CAS	Chemical Abstracts Service
CFU	colony-forming units
EC	European Commission and Enzyme Commission
EFSA	European Food Safety Authority
EINECS	European Inventory of Existing Commercial chemical Substances
EU	European Union
FAIM	food additives intake model
FAO	Food and Agriculture Organization of the United Nations
HACCP	hazard analysis and critical control points
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
NIH	National Institute of Health
OECD	Organisation for Economic Cooperation and Development
*p*NA	*p*-nitroaniline
TOS	total organic solids
TSEs	transmissible spongiform encephalopathies
WHO	World Health Organization