

**Opinion of the Scientific Panel on Food Additives,
Flavourings, Processing Aids and Materials in Contact with Food
on a request from the Commission related to**

**Use of an enzyme preparation based on thrombin:fibrinogen derived from
cattle and/or pigs as a food additive for reconstituting food**

Question number EFSA-2004-025

Adopted on 26 April 2005

SUMMARY

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food has been asked to issue an opinion on the safety in use of an enzyme preparation based on thrombin and fibrinogen derived from cattle and/or pigs as a food additive for reconstituting food.

The enzyme preparation consists of thrombin (EC 3.4.21.5) and fibrinogen, both obtained from blood plasma. The thrombin:fibrinogen preparation is applied to meat where thrombin transforms fibrinogen to fibrin that interacts with collagen enabling the binding of meat pieces in re-constituted meat. It can also be applied on poultry, fish and seafood.

As thrombin and fibrinogen are derived from edible parts of animals, no toxicological tests are required. This thrombin:fibrinogen preparation is produced from plasma obtained from blood of cattle or pigs, hygienically collected in slaughterhouses under veterinary inspection. For many years, several meat products with added animal blood or plasma have been produced and consumed in different countries without adverse effect. The applied concentrations of this thrombin:fibrinogen preparation are in the same range as the concentrations of these proteins in meat products with added blood or plasma. Any remaining thrombin would be partially inactivated by antithrombin III (also present in this thrombin:fibrinogen preparation). In addition any residual thrombin would be further inactivated during cooking (poor stability with heating) and in the stomach after consumption (low pH conditions). Consumption of meat products containing this enzyme preparation are not likely to increase the risk of allergic or intolerance response.

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food concluded that the use of the enzyme preparation based on thrombin:fibrinogen derived from cattle and/or pigs and produced as outlined in the opinion as a food additive for reconstituting food is not of concern from the safety point of view.

KEY WORDS

Thrombin, fibrinogen, fibrin, proteolytic enzyme, blood plasma, reconstituted food

BACKGROUND

An enzyme preparation based on thrombin and fibrinogen and derived from blood plasma of cattle and pigs (fibrinogen and thrombin) is being used by the meat industry for re-constituting fresh meat to achieve meat of desirable size and form.

This preparation is produced by a patented process and contains fibrinogen and thrombin obtained from blood plasma. The plasma is obtained from blood of cattle or pigs that is hygienically collected in slaughterhouses. This thrombin:fibrinogen preparation is applied to meat where thrombin transforms fibrinogen to fibrin. Fibrin interacts with collagen enabling the binding of meat pieces resulting in re-constituted meat. It can also be applied to poultry, fish and seafood.

In July 2001, the Standing Committee for Foodstuffs confirmed the legal status of this thrombin:fibrinogen preparation. The use of fibrinogen and thrombin leading to the formation of fibrin that has a function of binding meat pieces together fulfils the definition of a food additive laid down in Article 1(2) of Directive 89/107/EEC.

Categories of food additives are listed in Annex I of Directive 89/107/EEC and in Directive 95/2/EC. The described use of this thrombin:fibrinogen preparation would fall under the category of “stabilisers”: *substances which make it possible to maintain the physico-chemical state of a foodstuff: stabilisers include...substances which increase the binding capacity of the food including the formation of cross-links between proteins enabling the binding of food pieces into reconstituted food.*

France has given a temporary authorisation for this enzyme preparation for two years under Article 5 (1) of Directive 89/107/EEC, starting from 6 December 2003. AFSSA (Agence Française de Sécurité Sanitaire des Aliments) has given opinions on its use in May 2001 and in February 2003 (AFSSA 2001, 2003).

TERMS OF REFERENCE

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food is asked to issue an opinion on the safety in use of an enzyme preparation based on thrombin and fibrinogen derived from cattle and/or pigs as a food additive for reconstituting food.

ASSESSMENT

TECHNICAL DATA

Active components

Thrombin (EC 3.4.21.5.) is a serine proteinase involved in the final step of blood coagulation (Barrett et al., 2004). This enzyme consists of two chains and has an apparent molecular mass of about 36.5 kDa and an isoelectric point of 7.5 (Barrett et al., 2004). Thrombin has a highly specific proteolytic activity, cleaving only certain arginine-glycine peptide bonds such as those found in fibrinogen which is its most abundant natural substrate (Stryer, 1995). Thrombin is most active at pH 8 in the presence of at least 0.1 M NaCl (Orthner and Kosow, 1980, Barrett et al., 2004). The thrombin activity is expressed as NIH-U (National Institute of

Health-Units) and is measured with a chromogenic substrate. One bottle of commercial thrombin solution (100 mL) contains approximately 20 mg protein (3% of this protein would correspond to pure thrombin according to the petitioner) with an activity equivalent to about 2000 NIH-U.

The thrombin solution is always used in combination with a fibrinogen concentrate. The combination of both preparations is commercialised under the name Fibrimex. The main reaction product after using thrombin in combination with fibrinogen is fibrin. The released fibrin monomers having a different surface-charge pattern lead to their specific aggregation forming an insoluble fibrin network (Stryer, 1995).

Specifications

The enzyme preparation is based on thrombin (enzyme) and fibrinogen (substrate) solutions that are mixed together just before use. The respective specifications are as follows:

Thrombin

Chemical data	Protein	0.02% (w/v)
	Thrombin	3% (w/w) of protein
	Water	92-94% (w/v)
	Ash	6-7% (w/v)
	Total organic solids	< 1% (w/v)
Heavy metals	Arsenic	< 0.2 mg/kg
	Cadmium	< 0.1 mg/kg
	Mercury	< 0.2 mg/kg
	Lead	< 0.1 mg/kg
Physical data	pH	6-9
	Appearance	Colourless solution
	Taste	Salty
	Smell	Neutral
Microbiological data	Total bacterial count	< 100000 cfu/g
	<i>Salmonella</i> spp	negative/25 g
	<i>Staphylococcus aureus</i>	< 1000 cfu/g
	Enterobacteriaceae	< 1000 cfu/g
	Moulds	< 100 cfu/g
	Yeasts	< 100 cfu/g

Fibrinogen

Chemical data	Protein	9.5 % (w/w)
	Fibrinogen	5 % (w/w)
	Dry matter	11 % (w/w)
	Citrate	0.5 % (w/w)
	Fat	0.3 % (w/w)
Heavy metals	Arsenic	< 0.2 mg/kg
	Cadmium	< 0.1 mg/kg
	Mercury	< 0.2 mg/kg
	Lead	< 0.1 mg/kg
Physical data	pH	7-9
	Appearance	Viscous orange/red solution
	Taste	Characteristic
	Smell	Neutral
Microbiological data	Total bacterial count	< 100000 cfu/g

<i>Salmonella</i> spp	negative/25 g
<i>Staphylococcus aureus</i>	< 1000 cfu/g
Enterobacteriaceae	< 1000 cfu/g
Moulds	< 100 cfu/g
Yeasts	< 100 cfu/g

Source materials

According to the petitioner, thrombin and fibrinogen are isolated from cattle and pig blood from veterinarian inspected approved animals at EU licensed slaughterhouses. The blood is hygienically collected and is processed in hygienically designed equipment and stored at 4°C. The blood is separated into plasma and bloodcell concentrate. The plasma constitutes the raw material for thrombin and fibrinogen preparation. Production is under supervision of the national veterinary inspection authorities. According to the petitioner, no pathogenic microorganisms have been detected. Antibiotic activity was not detected in thrombin solution. The assays were based on agar medium inoculated with *Bacillus subtilis* BGA at pH 6.0, 7.2 and 8.0, and agar medium inoculated with *Micrococcus luteus* at pH 6.0 and 8.0. As a consequence the addition of thrombin does not cause an increase in the total microbial count in the foodstuff.

Manufacturing process

The manufacturing processes for thrombin and fibrinogen are described by the petitioner and consist of the following stages (Paardekooper and Wijngaards 1986):

Thrombin production involves 3 steps: isolation of prothrombin from plasma, purification by ion exchange chromatography and activation to thrombin. The blood is stored at 4°C and separated into blood cells and blood plasma. The liquid plasma is the raw material for thrombin. Prothrombin is separated by ion-exchange chromatography using a DEAE-Sephrose FF column and a 2 M sodium chloride (NaCl) solution with 0.01 M sodium citrate as eluent. A solution consisting of 0.2 M NaCl in 10 mM sodium citrate is used to remove any unbound plasma proteins from the column. Prothrombin, diluted 20 times in water, is activated by meat thromboplastin from a meat extract in the presence of 0.6 M calcium chloride solution. The meat extract is made from shoulder muscles of beef or pork that are homogenised 1:4 in 0.12 M sodium phosphate solution and centrifuged. The supernatant is collected and used as meat extract. Activation of thrombin is dependent on the free calcium ion level (Manseth *et al.*, 2003). The obtained solution is reported by petitioner to contain 20 NIH-U/ml of thrombin, 0.05 M NaCl, 0.5 mM sodium citrate and 0.6 M CaCl₂.

Fibrinogen is produced from blood that is collected as previously described for thrombin production. It involves precipitation by adjusting the temperature. The blood is separated into blood cells and blood plasma. The plasma is frozen at -3°C for a maximum of 7 days and then melted at 0°C and centrifuged for the separation of fibrinogen. The level of fibrinogen is standardized to 5% (w/w) by the addition of plasma, sieved with a 350 micrometer screen; the solution is filled into bags and kept under frozen storage (lower than -18°C).

CARRIERS AND OTHER ADDITIVES AND INGREDIENTS

All salts used in the isolation process are food-grade. No enzyme immobilisation procedures are performed.

USAGE

Thrombin is used to convert fibrinogen into a fibrin gel that binds meat pieces. The foodstuffs in which the enzyme is intended to be used by the petitioner are meat, poultry, fish and seafood.

The percentage of thrombin solution used by the petitioner in the final food application is 0.25-0.50% (vol/w). The thrombin activity of 50-100 NIH-U/kg in the meat directly after preparation is equivalent to a content of 0.5-1.0 mg protein/kg meat. Fibrinogen is added keeping a thrombin:fibrinogen ratio within the range 1:20 to 1:10. Thrombin and fibrinogen solutions are thawed before use; then, mixed for a few seconds and immediately (less than 8 minutes) added to the meat pieces by mixing or spraying. The final product is fibrin that interacts with collagen enabling the binding of meat pieces resulting in re-constituted meat with the required form, shape or casing. The re-constituted meat exhibits a lower cooking loss after frying or roasting. In general, as more thrombin is added, the processing time is shorter and the binding strength increases. However, the binding strength depends on several additional factors like pH (optimum pH is 7.0), meat temperature, moisture, size of meat cuts (stronger binding for larger particle size) and direction of meat fibres (optimum if the fibrin gel runs parallel to the available collagen in meat) (Boles and Shand, 1998; Chen and Lin, 2002). The use of previously frozen meat has no effect on the binding process (Boles and Shand, 1999).

Stability and fate in the food

Thrombin stability is rather poor. Once the binding process is achieved (overnight), no residual thrombin activity can be detected at a detection limit of 0.004 NIH-U/ml. Thrombin activity decreases very rapidly with increases in temperature (i.e. half-lives of 60, 35 and 6 min at 25, 37 and 45°C, respectively) (Le Borgne and Graber, 1994).

EVALUATION OF SAFETY IN USE

As thrombin and fibrinogen are derived from edible parts of animals, no toxicological tests are required (SCF, 1992). These thrombin:fibrinogen preparations are plasma protein fractions prepared from cattle and/or pig plasma using a physical procedure without added components. The applied amounts of the enzyme preparation result in protein concentrations comparable to those in meat products with added blood or plasma. For many years, several meat products with added animal blood or plasma have been produced and consumed in several countries without any known indications that these products exhibit any increased allergic or intolerance response after consumption. Furthermore, the fibrin gel is well degraded by proteases (Kolev et al., 1996; 1997) and can be expected to be degraded by enzymes in the intestinal tract after consumption. Consumption of foods containing the enzyme thrombin are not expected to give rise to problems of safety since it is derived from edible parts of animals. Any remaining thrombin would be partially inactivated by antithrombin III (Borsodi and Machovich, 1979) (also present in this thrombin:fibrinogen preparation). In addition any residual thrombin would be further inactivated during cooking (poor stability with heating) (Le Borgne and Graber, 1994) and in the stomach after consumption (low pH conditions). Furthermore according to the petitioner no thrombin

activity (detection limit 0.004 NIH-U/ml) was detectable after overnight treatment. Consumption of meat products containing this thrombin:fibrinogen preparation are not likely to result in an increased risk for allergic or intolerance responses.

Thus, taking into account the above comments, the Panel concluded that the thrombin:fibrinogen preparation derived from cattle and/or pigs and produced as outlined in this opinion as a food additive for reconstituting food is not of concern from the safety point of view.

CONCLUSIONS AND RECOMMENDATIONS

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food concluded that the use of the enzyme preparation based on thrombin:fibrinogen derived from cattle and/or pigs and produced as outlined in this opinion as a food additive for reconstituting food is not of concern from the safety point of view.

DOCUMENTATION PROVIDED TO EFSA

The dossier submitted by the applicant contained:

Letter from the permanent Representation of France, including the provision for authorisation of the enzyme preparation derived from bovines/pigs in France, the opinion of AFSSA on the safety of the enzyme preparation and on the authorisation of the enzyme preparation.

Application from Harimex B.V., Loenen, The Netherlands.

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