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The Heat Stability of Some Trypsin Inhibitors in Meat Products with Special Reference to Added Soybean Protein*

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Summary. The heat stability of the trypsin inhibitors commonly present in meat products has been investigated. This includes the naturally occurring inhibitors in meat or serum, and some of the soybean proteins usually added to minced meat and sausages. The stability of the inhibitors are investigated both alone, and when mixed with meat and other compounds.

To test the inhibitory activity two methods were used. For quantitative measurement the Kunitz method was applied. For qualitative and semiquantitative investigation of inhibitory activity the casein precipitating method of *Sandvik* was adapted. The latter method is more sensitive than the first mentioned.

Of the animal sera investigated in the present work the inhibitors in horse serum revealed the highest thermostability. After heating for 5 min at 100° C, the trypsin inhibitory activity was 10% of the original, while the inhibitory activity in the other animal sera investigated was lost after exposure for 5 minutes at 80—85° C. The inhibitors in the various soybean proteins were far more stable. The heat stability was shown to be pH-dependent.

The inhibitory activity of the various soybean proteins was, however, destroyed when heating was carried out in the presence of meat or meat extract at 80—85° C for 2—5 min. Substances added to the minced meat, such as starch, casein, milkpowder or skimmed milk, and spices did not have this effect on the heat stability of the soybean inhibitors. It was further shown that the factor in meat responsible for decreasing the thermostability of the trypsin inhibitors in soybeans is heat labile, it was precipitated by ammonium sulphate and was a high molecular weight compound as shown by gel chromatography.

The significance of this phenomenon is discussed and a possible explanation is put forward.

Zusammenfassung. Die Hitzestabilität der üblicherweise in Fleischprodukten vorhandenen Trypsininhibitoren wurde untersucht. Dies schließt die natürlichen Vorkommen von Inhibitoren in Fleisch oder Serum ein und einiger Sojaproteine, die üblicherweise Fleischbrät und Würsten zugesetzt werden. Die Stabilität der Inhibitoren wurde sowohl für sich als auch mit Fleisch und anderen Verbindungen untersucht. Um die Hemmaktivität zu testen, wurden 2 Methoden angewendet; für quantitative Messungen die Kunitz-Methode, für qualitative und halbquantitative Untersuchungen die Casein precipitierende Sandvik-Methode, wobei die letztere Methode empfindlicher ist als die erste. Von den untersuchten tierischen Seren besaß der Inhibitor im Pferdeserum die höchste Thermostabilität. Nach 5 min Erhitzung auf 100° C war die trypsininhibitorische Wirkung nur noch 10% der ursprünglichen, während die der anderen tierischen Seren nach 5 min bei 80—85° C vernichtet wurde. Die Inhibitoren der verschiedenen Sojabohnenproteine waren weit stabiler. Die Hitzestabilität ist pH-abhängig. Die inhibitorische Aktivität der verschiedenen Sojabohnenproteine wurde jedoch zerstört, wenn die Erhitzung in Gegenwart von Fleisch oder Fleischprodukten bei 80—85° C für 2—5 min ausgeführt wurde. Zu Fleischbrät zugesetzte Substanzen wie Stärke, Casein, Milch- oder Magermilchpulver und Gewürze haben keinen Einfluß auf die Hitzestabilität des Sojabohneninhibitors. Es wurde weiter gezeigt, daß der Faktor, der in Fleisch für die Abnahme der Thermostabilität des Trypsininhibitors in Sojabohnen verantwortlich ist, hitzelabil ist; er wurde durch Ammoniumsulfat ausgefällt und wies ein hohes Molekulargewicht, belegt durch Gelchromatographie, auf. Die Signifikanz dieser Phenomene wird diskutiert und eine mögliche Erklärung dafür gegeben.

Introduction

Naturally occurring trypsin inhibitors have been found in various materials of vegetable and animal origin [1]. It has been suggested that some of these inhibitors cause growth retardation, pancreatic hypertrophy, and lead to decreased food utilization when present in an active state in animal feed. The importance of heating soybean protein in order to destroy the trypsin inhibitory activity has been reported by numerous investigators [2—6].

There is great divergence in the results concerning the thermostability of the soybean trypsin inhibitors. The discrepancies may be partly due to the fact that soybeans contain several different inhibitors, which vary in stability to heat, stability to acid, digestibility by pepsin, as well as in molecular weight, cysteine content and inhibitory spectrum. Thus, the Kunitz inhibitor is reported to be unstable to heat, while the Bowman-Birk inhibitor is heat stable. Secondly, the amounts and

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relative proportions of the various inhibitors in different soybean varieties differ, and variations also occur during the maturation process. Thirdly, the conditions under which the experiments are performed are of importance, particularly moisture and pH. The terms "heat labile" and "heat stable" are also not precisely defined.

Soybean proteins are added to various meat products, such as minced meat and sausages. Preliminary investigations have shown that the inhibitors present in soybean protein were more heat stable than those present in meat [7]. The posibility was therefore considered of detecting inhibitory activity in processed products, such as sausages, due to added soybean protein, and thus use the more heat stable inhibitors as indicators for the presence of soybean protein. Kotter *et al.* [8] reported, however, that trypsin inhibitory activity could not be detected in gently heated Frankfurter-type sausages containing soybean protein. The question arose, therefore, as to whether factors in minced meat could influence the heat stability of the soybean trypsin inhibitors.

The aim of the present work was to study the heat stability of the trypsin inhibitors commonly present in meat products, such as the naturally occurring inhibitors in meat or serum, and some of the soybean proteins used, both alone and when mixed with meat and other compounds.

Materials and Methods

Enzymes and Inhibitor-Containing Proteins. Trypsin from hog pancreas (crystallized, batch No. 36467) was obtained from Koch-Light[1]. Soybeam trypsin inhibitor (SBTI) (Type II-S, crude lot 56B-1970) was obtained from Sigma[2]. The following brands of commercial soybean proteins were tested. Promine D from Central soy[3], Soyamin-90 from Lucas Meyer[4], and Ipso Mr from Fuju Oil[5]. Soybeam flour B (Tanzania B) was obtained from a village in Tanzania (for Ref. see Holm *et al.* [9]).

Extracts of the soybean proteins were prepared by making 2.5 or 10% suspensions in saline, or in a phosphate buffer, as indicated in each case. The suspensions were left overnight at 4° C and filtered before use. Extracts of minced meat with, or without, commercial soybean protein and meat extracts were prepared by taking two parts of the meat material and one part of saline. After thorough blending, the mixtures were left overnight at 4° C, and centrifuged at 15.000 g for 20 min. The supernatant liquide were kept at 4° C, or frozen, until used. Whole blood samples of cattle, horse, pig and sheep were allowed to coagulate, and the serum was collected after centrifugation. It had previously been shown that storage of sera from the animal species tested in this investigation for some months at low temperatures did not influence the inhibitory activity [10]. In the heating experiments, the sera were diluted 1 : 2 or 1 : 4 with saline.

Merthiolate was usually added to all extracts and sera to a final concentration of 1 : 10.000.

Heating. The material to be heated was transferred, in aliquots of 0.2 ml, to thin-walled 1 ml glass ampoules. After sealing, the ampoules were stored in an ice bath until heated. Heating was carried out in a Haake ultrathermostat containing glycerol. During heating the ampoules were completely submerged in the glycerol bath, after which they were rapidly cooled in an ice bath.

Assay of Inhibitory Activity. A slight modification of the casein digestion method of Kunitz [11], as previously described by Fossum [12], was used to determine the activity of the inhibitors against swine trypsin. 0.3 ml of 0.5 M Tris-HCl buffer, pH 7.0, and distilled water were added to the tubes to give a final volume of 1 ml, after addition of the enzyme in the absence, or presence of inhibitor-containing materials. The enzyme-containing solution without inhibitors, with non-heated inhibitors, or with inhibitory material exposed to various degrees of heat treatment, was routinely incubated in a water bath for 2 min at 37° C, after which the substrate (1 ml of a 2% solution of casein of Hammersten quality in 0.1 M phosphate buffer, pH 7.6) was added to start the reaction. After 20 minutes at 37° C, the reaction was stopped by adding 3 ml of a 5% solution of trichloroacetic acid (TCA). The activivity of the enzyme was determined by reading the absorbance of the supernatant liquid at 280 nm using a Beckman DU spectrophotometer. The percent inhibition caused by various amounts of the inhibitor-containing materials was calculated for each series of unheated and heated material.

An amount of trypsin (0.025 ml of a 0.1% solution) resulting in a final optical density change of 0.500—0.600 in the absence of inhibitor under the conditions described, was used.

The inhibitory activity was also tested by the agar casein precipitating method (CP-method) of Sandvik [13] adapted for the determination of proteinase inhibitors, as in the crosswise casein precipitating inhibition test (crosswise CPI-test) [12]. Filter paper strips (Schleicher & Schüll, No. 2043) moistened with the inhibitor-containing material were placed on an agar layer (2 mm deep) containing 1% sodium caseinate (pH 6.5) in 1.4% agar (Bacto-Agar, Difco), with 0.01% merthiolate and 0.003 M $MgCl_2$, and incubated for about 3 h at 37° C. After removal of these strips, similar strips moistened with a solution of swine trypsin were applied to the surface of the agar at right angles to the direction of application of the inhibitors. The tightly covered plates were incu-

[1] Koch-Light Laboratories Ltd. Colnbrook, Buckinghamshire, England.
[2] Sigma Chemical Company, St. Louis, Mo., U.S.A.
[3] Central soya, 66 Rue Royal 1000 Brüssel.
[4] Lucas; Meyer, Hamburg 28, Germany.
[5] Fuju Oil, Osaka, Japan.

bated at 37° C for 6—18 h. Proteolytic activity is indicated by white zones in the clear agar, while inhibition is indicated by interruption of the white precipitation zones, or by a certain degree of narrowing of the white zones, depending on the inhibitory activity, in the region where application of inhibitor and enzyme overlap (Figs. 1 and 4). Enzyme concentrations corresponding to 10—100 CP-units per 0.025 ml were usually used [13].

The inhibitory activity was tested immediately after the series in each case was heated and cooled.

Results

The trypsin inhibitory activity of various animal sera after heating for 5 minutes at different temperatures, as tested by the crosswise CPI-test, is shown in Fig. 1. It can be seen that after heating for 5 min at 100° C there is still inhibitory activity in the horse serum, while the inhibitors in the other animal sera tested are all inactivated at 85° C.

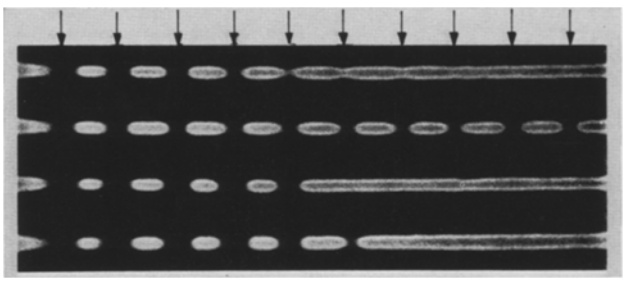

Fig. 1. The effect of heating upon the trypsin inhibitory activity in sera from various animal species as tested by the crosswise casein precipitation inhibition test (Crosswise CPI-test). From left to right the sera, previously diluted 1 : 4 were heated as follows: unheated, heated at 60°, 65°, 70°, 75°, 80°, 85°, 90°, and 100° C for 5 min. The animal were (downwards): pig, horse, cattle and sheep. The enzyme used was swine trypsin (0.005 mg per ml)

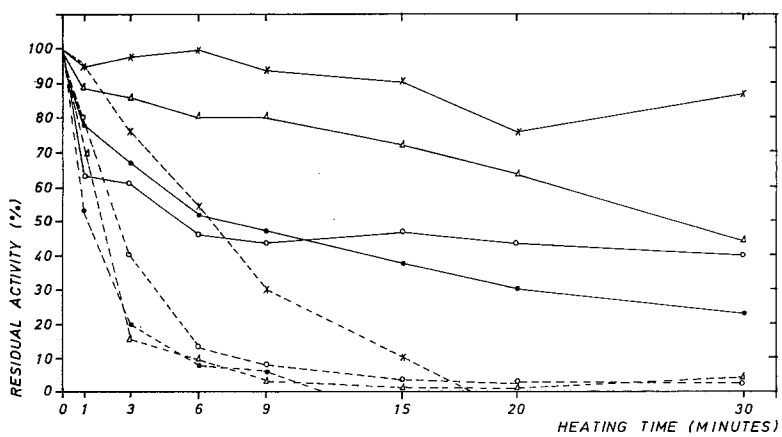

Fig. 2. The effect of heating for various periods of time at 100° C upon the trypsin inhibitory activity in Tanzania B (5 mg per ml) (x———x), SBTI (0.5 mg per ml) (△———△), Promine D (50 mg per ml) (○———○), and Ipso MR (50 mg per ml) (●———●), at pH 6 (whole lines) and pH 9 (interrupted lines) as tested by the Kunitz method. Solutions of the inhibitor-containing material were made in 0.2 M phosphate buffer. The residual inhibitory activity is expressed as per cent of the inhibitory activity in the unheated materials

By the Kunitz method it was found that the inhibitory activity in horse serum, after heating at 100° C for 5 min was 10% of the original.

Fig. 2 shows the effect of heating for various periods of time at 100° C upon the trypsin inhibitory activity of four different soybean materials as tested by the Kunitz

method. The heating was carried out in 0.2 M phosphate buffer at pH 6.0 and 9.0. The remaining inhibitory activity is expressed as per cent of the trypsin inhibitory activity in the unheated material. It can be seen that the inhibitors in all the samples are far more stable at pH 6.0 than at pH 9.0. It can also be seen that the stabilities of the inhibitors in the various materials differ considerably, the inhibitors in Tanzania B soybean being more stable at both pHs than the inhibitors in the other samples of soybean protein tested.

The influence of the inhibitory activity of SBTI upon trypsin after heating for various periods of time at different temperatures is shown in Fig. 3. Inhibitory activity is still present after heating for 30 min at 120° C, while after heating for more than 15 min at 130° C no inhibitory activity could be demonstrated by the Kunitz method. Tested by the crosswise CPI-test, the activity of inhibitors could still be detected after a heat treatment of 20 min at 130° C.

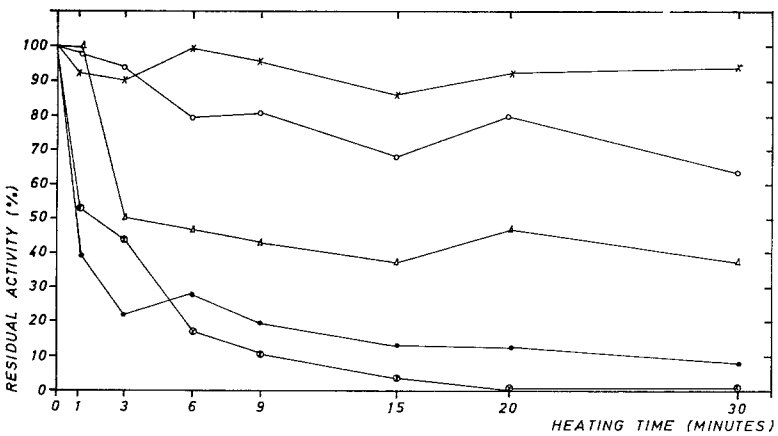

Fig. 3. The effect of heating for various periods of time at 40° C (x———x), 60° C (○———○), 100° C (△———△), 120° C (●———●), and 130° C (⊗———⊗) upon the trypsin inhibitory activity of SBTI (0.5 mg per ml in saline) as tested by the Kunitz method. The residual inhibitory activity is expressed as per cent of the inhibitory activity in the unheated SBTI-solution

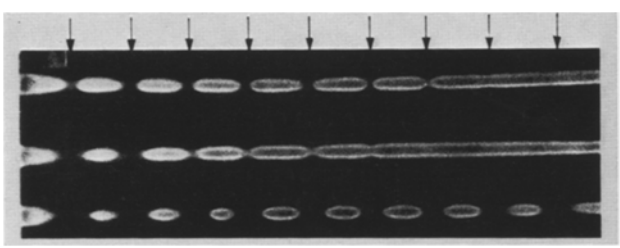

Fig. 4. The effect of heating at various temperatures on the inhibitory activity in (downwards): Minced meat, minced meat with 2% Promine D, and a 2% solution of Promine D in saline (pH 6.4) as tested by the crosswise CPI-test. From left to right as marked by arrows, the materials were heated as follows: unheated, heated at 60°, 70°, 75°, 80°, 85°, 90°, 95°, and 100° C for 5 min. The enzyme used was swine trypsin (0.005 mg per ml)

The effect of heat treatment at various temperature on the inhibitory activity in minced meat (made from bovine meat), in minced meat with 2% Promine D added, and in a 2% solution of Promine D, as tested by the crosswise CPI-test, is shown in Fig. 4. It can be seen that no inhibitory activity could be demonstrated in extracts from minced meat after heating for 5 minutes at 95° C. The inhibitors in the same

minced meat product, but with Promine D added, were even less heat stable than those in minced meat without Promine D, while the inhibitory activity in Promine D alone was not influenced by heating for 5 min at 100° C, (as far as can be demonstrated by this method under the conditions used.)

The same results as were obtained with Promine D, were also observed following containing heat treatment of minced meat IPSO MR, Soyamin, Tanzania B and commercial SBTI, as well as heat treatment of solutions of these soybean proteins.

Substances added to the minced meat, such as starch, casein, milk powder or skimmed milk, and spices did not have any effect in the heat stability of the soybean inhibitors. When extracts from ground cattle meat, however, were added to solutions of the soybean proteins, the thermostability of the inhibitors decreased. Ground meat from various animals, such as horse, pig, sheep, hen, rabbit and also various fish species had the same effect upon the thermostability of the soybean inhibitors as had bovine meat. Blood serum from the same animals did not show this effect.

By the Kunitz method it was shown that the inhibitory activity in unheated mixtures of the various soybean products and ground, or minced meat, was equal to the sum of the inhibitory activity in each of the components.

By chromatography of meat extract on a Sephadex G-100 column it was shown that the factor responsible for decreasing the thermostability of the soybean trypsin inhibitors was eluted in the void volume. The factor could be precipitated by ammonium sulphate. Heating the factor at 80° C for 5 minutes lead to inactivation of the thermostability-destroying effect.

Discussion

The present investigation shows 1) that there are great differences in the thermostability of the naturally occuring inhibitors present in various sources, and 2) that the thermostability of the inhibitors is dependent upon environmental conditions. Of special interest is that the thermostability of the inhibitors in soybean protein are so markedly influenced by the presence of certain proteinous compounds present in meat. Many of the naturally occuring proteinase inhibitors are remarkably resistant to heat [14–20]. Since meat contains appreciable amounts of trypsin inhibitory compounds from serum, it was considered important to determine the thermostability of these inhibitors. Of the animal sera investigated in the present work, the inhibitors in horse serum revealed the highest thermostability. After heating for 5 min at 100° C the trypsin inhibitory activity was 10 % of the original. The trypsin inhibitory activity of the other animal sera investigated was lost after heating for 5 min at 80° C (cattle) and 85° C (pig and sheep). The trypsin inhibitors found in meat are considered to be the same as those found in serum, based on electrophoretic, inhibitory spectrum and thermostability studies [10]. It was also confirmed by Nordal and Fossum [7] that the inhibitors in meat of bovine origin were destroyed by boiling for a short time. For human serum, Shulman [21] found that the inhibition of trypsin (and chymotrypsin) decreased to 10 % of the original value in approximately 20 min at 60° C. When diluted serum (1 : 10) was heated at 100° C, the trypsin and chymotrypsin inhibitory activity decreased to approximately 5 % within 10 minutes. Some inhibitory activity could be detected even after 30 minutes of heating at 100° C [21].

Soybeans contain different proteinase inhibitors, which vary considerably in properties, as for instance, heat stability [1, 22]. In the present work the thermostability of proteinase inhibitors in various crude soybean proteins has been investigated. It was confirmed that, after boiling for several minutes, much of the inhibitory activity was still present. The inhibitors were more stable when heated in neutral surrounding than when exposed to heat at higher pHs. Thus, for the two commercial soybean proteins investigated, approximately 45 % of the original activity was retained after heating at 100° C for 10 min at pH 6.0. The inhibitors in Tanzania B and

SBTI materials were far more stable. Wallace *et al.* [23] also found that the inactivation of the trypsin inhibitor increased when increasing the pH from neutrality to 9.9.

Soybean proteins are often used as stabilizing agents in the production of various meat products. The small amounts added (1–2%) contribute to a considerable increase in the inhibitory activity of the raw product. Due to the thermostability of the inhibitors, it could be possible that the inhibitors under certain conditions, might lead to alimentary disturbances. It was shown, however, that the presence of inhibitors in heated products could not be used as an indicator for the addition of soybean products. After gentle heating (90° C for 5 min) of a mixture of meat and soybean protein, no inhibitory activity could be detected by use of the casein precipitation inhibition test (CPI-test), which has been found to be very sensitive for the determination of inhibitory activity [12].

This result is in agreement with Kotter *et al.* [8], who concluded that "owing to technological influences, however, no activity on the part of trypsin inhibitors could be detected in canned Frankfurther-type sausages, even where 1.5% soya protein (Promine D) was added and the products were gently heated". The difference in the heat stabilities of the soybean protein inhibitors in the absence, and in the presence, of meat and meat extracts is enigmatic.

Biological material, like enzymes and inhibitors, are usually more stable against heat in the presence of other proteins than in a pure state. The fact that serum or casein did not have this effect indicated a special mechanism of action. The chromatography studies, the observation that the factor responsible for the effect was precipitable by ammonium sulphate, and the heat lability of the factor, indicated that it was a proteinous component. A possible explanation for the phenomenon may be that the heat stable soybean inhibitors, for example the Bowman-Birk inhibitor, contain several disulfide bonds in the molecule. These apparently stabilize the molecule [22]. Some animal organs, for instance meat, contain protein rich in SH-groups. It is possible that these groups interact with the disulphide bonds in the inhibitor, and thus render the inhibitor molecule more sensitive to heat.

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