Trypsin Inhibition by Free Fatty Acids and Stearoyl-CoA†

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Potassium salts of long-chain fatty acids inhibited trypsin. The longer the chain, the stronger the inhibition. Among the C_{18} fatty acids, oleate was the most effective inhibitor. Stearoyl-CoA was 25 times more effective in inhibiting trypsin than stearate. Trypsin inhibition by fatty acids was partial and time and temperature dependent. Moreover, the inhibition was found to be a function of the ionization state (solution pH) of the inhibitory compounds. The presence of Ca^{2+} during preincubation of the inhibitor with trypsin protected the enzyme from inhibition. Inhibition appeared to be due to direct binding of the ionized fatty acid monomer to the enzyme rather than to a detergent effect of the inhibitory compounds.

Free fatty acids and their acyl-CoA esters reportedly inhibit various enzymes: glycolytic (Webber et al., 1966; Lea and Webber, 1968), gluconeogenic (Parvin and Dakshinamurti, 1970), lipogenic (Korchak and Masoro, 1964; Triscari et al., 1968), and others (Srere, 1965; Lin et al., 1976; Ito et al., 1987). Certain general characteristics of enzyme inhibition by fatty acids are the following: (i) the inhibition is time dependent, (ii) the longer the fatty acid chain, the stronger the inhibition, and (iii) the fatty acid CoA ester is a more effective inhibitor than its corresponding fatty acid.

Trypsin inhibition by fatty acids was first reported by Bargoni (1960). Later, Wang et al. (1975) found that free fatty acids were responsible for increased trypsin inhibition in fermented soybeans. In this study, certain new aspects of trypsin inhibition by free fatty acids and stearoyl-CoA were investigated. An inhibition mechanism was proposed. And finally, the trypsin inhibition by different free fatty acids was compared in terms of chain length and degree of unsaturation.

MATERIALS AND METHODS

Reagents. Butyric, hexanoic, octanoic, lauric, myristic, palmitic, stearic, and oleic acids were purchased from Fisher Scientific (Pittsburgh, PA). Capric, arachidic, linoleic, and linolenic acids, benzoyl-dl-arginine *p*-nitroanilide hydrochloride (BAPA), and crystalline bovine trypsin (Type III, 10 100–13 000 BAEE units/mg of protein, salt free) were supplied by Sigma (St. Louis, MO). The water used in this study was doubly distilled.

Buffer and Solutions. The assay buffer contained 10 mM $CaCl_2$ and 50 mM Tris, pH 8.2. Trypsin solution (16 μg/mL) was prepared with 0.001 N HCl solution. A stock BAPA solution was prepared by dissolving 400 mg of BAPA in 10 mL of dimethyl sulfoxide. A working BAPA solution (0.92 mM) was made by diluting the stock solution with the assay buffer prewarmed at 37 °C.

A stock solution of the potassium salt of each fatty acid was prepared by adding 0.5 N KOH to a mixture of water and fatty acid, while stirring and heating, if necessary, until pH 10 was reached. No KOH was added to the solution of stearoyl-CoA. A working solution of a desired concentration was prepared by diluting the stock solution with water (dilution factor varied from 25 to 160 with fatty acid). The concentrations of the stock and working solutions for each fatty acid and stearoyl-CoA are shown in Table I.

Trypsin Inhibition Assay. Portions (0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 mL) of each working fatty acid solution were pipetted into a set of test tubes and adjusted to 1.0 mL with water. A 10-min preincubation at 37 °C was started by adding 0.5 mL of bovine trypsin to each test tube. Two milliliters of BAPA solution was then added to start the enzymatic reaction. This brought the pH of the reaction mixture to 8.1 ± 0.2. The reaction was allowed to proceed for 10 min and stopped by injecting 0.5 mL of 30% acetic acid with a 1-mL syringe. The absorption of the reaction mixture (4 mL) was measured on a spectrophotometer at 410 nm and used as an estimate of trypsin activity.

Trypsin Inhibition Calculation. For each test, A_{410} was plotted against inhibitor concentration. The slope of the linear portion of the curve was used to express the antitryptic activity (A_{410} per unit of inhibitory compound). Alternatively, trypsin inhibition was expressed as percentage of trypsin activity in the absence of inhibitor.

RESULTS

Inhibition Curve. The relationship between trypsin inhibition and concentration of palmitate or linoleate is shown in Figure 1. The results indicate that at lower ranges of concentration inhibition increased greatly with salt concentration and started leveling off when about 55% of the trypsin activity was inhibited. Beyond this point, an increase in fatty acid concentration caused little additional inhibition.

Preincubation Time. The time of incubating linoleate with trypsin before addition of substrate was found to affect the final inhibition value (Figure 2). When substrate, inhibitor, and trypsin were mixed together at the same time (zero preincubation time), no inhibition was observed. Inhibition increased sharply during the first 8 min of preincubation. Preincubation longer than 8 min gave little additional inhibition. For obtaining equilibrium data, in subsequent assays, a 10-min preincubation of inhibitor with enzyme was adopted.

Ionization State. At pH <7.5, the stock solution of linoleate was turbid. Upon addition of 0.5 N KOH, the turbidity started disappearing, and at pH 9.0, the solution was perfectly clear, indicating that linoleic acid was fully ionized (Cistola et al., 1988).

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Table I. Concentrations of Stock and Working Solutions of Potassium Salts of Fatty Acid and Stearoyl-Coenzyme A

name	acid code	concentration	
		stock soln, mM	working soln, μM
butyric acid	C4	81.60	650
hexanoic acid	C6	31.00	600
octanoic acid	C8	12.50	500
capric acid	C10	8.13	300
lauric acid	C12	2.50	100
myristic acid	C14	2.00	40
palmitic acid	C16	0.84	30
stearic acid	C18	0.83	20
arachidic acid	C20	1.25	15
oleic acid	C18:1	3.17	20
linoleic acid	C18:2	3.22	20
linolenic acid	C18:3	3.94	80
stearoyl-CoA		0.20	1.6

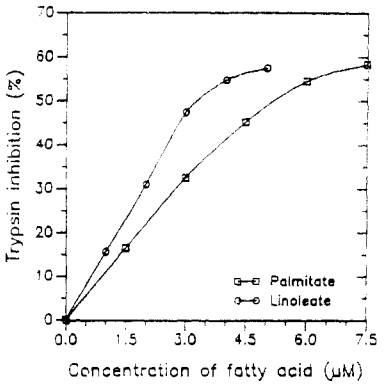

Figure 1. Trypsin inhibition by palmitate and linoleate as a function of inhibitor concentration.

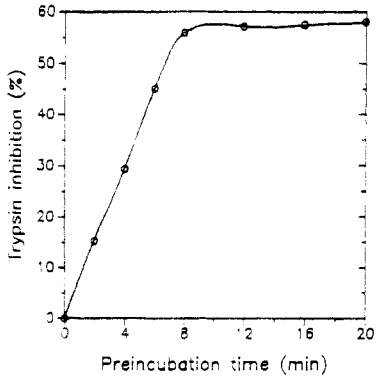

Figure 2. Effect of preincubation time on trypsin inhibition by linoleate. Bovine trypsin (8 μg) was preincubated with 20 nmol of linoleate in 1.5 mL of water at 37 °C for indicated periods. A 10-min enzymic reaction was started by adding 2.0 mL of BAPA (0.92 mM in the assay buffer) and stopped by adding 0.5 mL of 30% acetic acid. Controls were run for each time period by preincubating trypsin in the absence of linoleate.

Figure 3 illustrates the relationship between the trypsin inhibition and the pH of stock potassium linoleate solution (ionization state of linoleic acid). A slight increase in inhibition was observed as the pH increased from 4.0 to 8.0. As pH increased above 8.0, the inhibition increased sharply. It appeared that trypsin inhibition was mediated by the ionization state of the fatty acid.

Effects of Salts. The fact that mixing substrate, inhibitor, and trypsin at the same time resulted in no trypsin inhibition suggests not only that the inhibition depends on the time of interaction between the inhibitor and the enzyme but also that there is a protecting factor in the assay system, which is not present when the inhibitor is incubated with enzyme in the absence of substrate solution. In other words, the substrate solution may

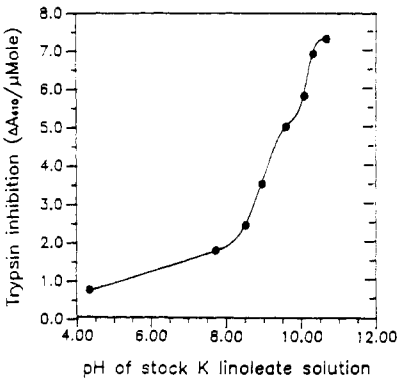

Figure 3. Trypsin inhibition by linoleate as a function of stock solution pH. The concentration of the stock linoleate solution was fixed at 3.22 mM regardless of pH. During assay, the solution was diluted into a working solution of 20 μM linoleate with water (1:160 dilution).

contain a protective factor. To confirm this observation, a separate study was conducted in which three assay procedures were used. In the first procedure, a 10-min preincubation of linoleate with trypsin was followed by addition of substrate dissolved in 2 mL of assay buffer. In the second procedure, a 10-min preincubation of linoleate with trypsin was followed by addition of 1 mL of assay buffer, and 10 min later, substrate was added in 1 mL of assay buffer. In the third procedure, a 10-min preincubation of linoleate with trypsin in the presence of 1.0 mL of assay buffer was followed by addition of substrate dissolved in 1 mL of assay buffer. It was found that the first and second procedures (preincubation in the absence of assay buffer) gave the same trypsin inhibition, while the third procedure (preincubation in the presence of assay buffer) resulted in no inhibition. The results indicate that (i) something in the assay buffer protected the enzyme from inhibition and (ii) protection occurred only before the interaction between the enzyme and the inhibitor. Once the interaction was complete, the protective agent was no longer effective.

As the assay buffer contained $CaCl_2$ and Tris, and the inhibitor solution contained K^+, a similar three-procedure study was conducted with solutions of individual salts, $CaCl_2$, Tris, and KCl, to find out which one was responsible for the protective effect of trypsin inhibition by fatty acids. The results showed that a 10-min preincubation in the presence of $CaCl_2$ at concentrations as low as 0.5 mM reduced trypsin inhibition to near zero (Figure 4). However, if $CaCl_2$ was replaced with either Tris or KCl, a much higher concentration of these salts was needed to cause the same extent of protection (Figure 5). In all cases, when added after preincubating linoleate with trypsin, none of these salts exhibited protective effects at any concentration.

Since KCl has been shown to protect trypsin from inhibition, did the K^+ of the fatty acid salts also protect trypsin from inhibition? The answer is no. The concentration of K^+ in the stock fatty acid solutions was about 15 mM, and in the assay mixture, 0.1–1 mM, which is far lower than the level leading to reduced inhibition.

Preincubation Temperature. A study was conducted in which the preincubation of linoleate with trypsin was carried out at 0 °C, while the enzyme activity assay (color reaction) was performed at the normal assay temperature (37 °C). Under these conditions, no trypsin inhibition was observed. This suggests that interaction of linoleate with trypsin could not occur at 0 °C and the inhibition was temperature dependent.

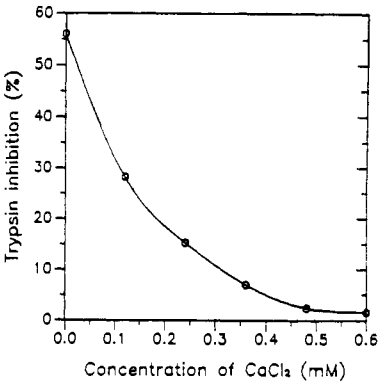

Figure 4. Effect of $CaCl_2$ on trypsin inhibition by linoleate. Bovine trypsin (8 μg) was preincubated with 20 nmol of linoleate in the presence of various levels of $CaCl_2$ in 2.5 mL of water at 37 °C for 10 min. The enzyme activity was tested with 1.0 mL of BAPA (1.84 mM in assay buffer).

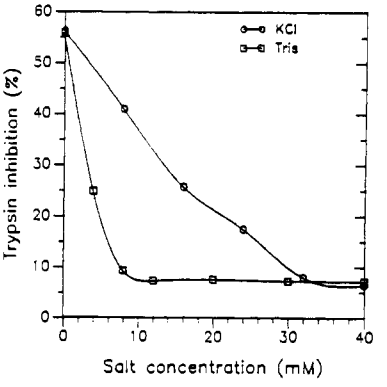

Figure 5. Effect of Tris and KCl on trypsin inhibition by linoleate. Bovine trypsin (8 μg) was preincubated with 20 nmol of linoleate in the presence of various levels of Tris or KCl in 2.5 mL of water at 37 °C for 10 min. The enzyme activity was tested with 1.0 mL of BAPA (1.84 mM in assay buffer).

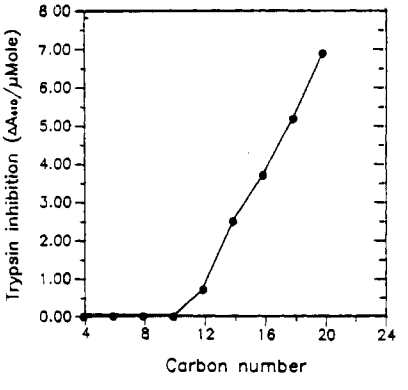

Figure 6. Trypsin inhibition by potassium salts of saturated fatty acids.

Comparison of Trypsin Inhibition by Different Fatty Acids. After the above parameters of trypsin inhibition by fatty acids were investigated, the inhibition values of various potassium salts of fatty acids were compared in terms of carbon chain length and degree of unsaturation. It was found that among the saturated fatty acids those with C-chain lengths of 4–10 showed no trypsin inhibition, and as chain length increased beyond 10, trypsin inhibition also increased (Figure 6). Among the C_{18} fatty acids, oleate exhibited the highest inhibition, but greater degrees of unsaturation resulted in lower inhibition. In fact, linolenate caused less inhibition than stearate (Figure 7).

Trypsin Inhibition by Stearoyl-CoA. Although studies have shown that acyl-CoA esters of fatty acids inhibit various enzymes (Srere, 1965; Ponda and Mead, 1970; Ito et al., 1987), their inhibition of trypsin has not been reported. In this study, the effect of stearoyl-CoA on trypsin activity was investigated as previously described except that it was added to the assay system as an acid and not as a potassium salt. Stearoyl-CoA had remarkable similarity in trypsin inhibition to the potassium salts of fatty acids in terms of time and concentration dependence and salt protection. However, the former showed higher inhibition capacity. Figure 8 is a trypsin inhibition curve of stearoyl-CoA. At concentrations ranging from 0 to 0.2 mM, the inhibition increased linearly. At concentrations higher than 0.2 mM, the curve leveled off. The inhibition value of stearoyl-CoA, calculated to be 131 A_{410}/μmol, was about 25 times that of stearate, 5.23 A_{410}/μmol.

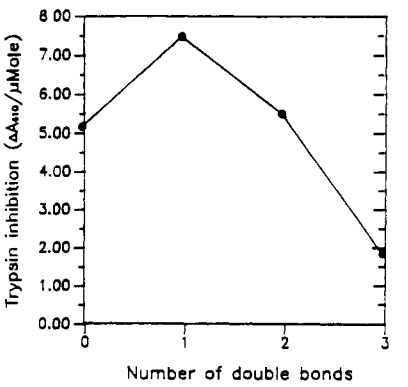

Figure 7. Trypsin inhibition by potassium salts of C_{18} fatty acids.

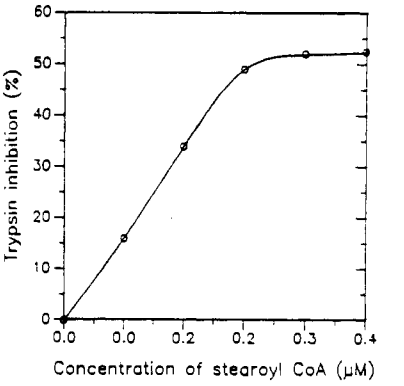

Figure 8. Trypsin inhibition as a function of stearoyl-CoA concentration.

DISCUSSION

One widely proposed mechanism of enzyme inhibition by a fatty acid and its acyl-CoA ester is via the so-called "detergent effect" (Parvin and Dakshinamurti, 1970; Pande and Mead, 1968). According to this hypothesis, the fatty acid or its ester is fully ionized at neutral pH and they form fatty acid micelles which interact with the enzyme. However, Lin et al. (1976) and Ito et al. (1987) disagreed with the above hypothesis and proposed a "monomer binding" theory; that is, the inhibitory effect of long-chain fatty acid is due to the monomeric form rather than micellar formation. This theory was further supported by the observation that the predominant phase of fatty acids in water at physiological pH and temperature is the lamellar fatty acid/soap phase, rather than the micellar phase (Cistola et al., 1988).

In this study, the solution pH of stock fatty acid salts was standardized at 10.0. According to Cistola et al. (1988), micelles should form in the stock solution at this pH value.

However, there was not formation of micelles in the working solution, because after dilution, (i) the pH dropped from 10.0 to 7.0 or less and (ii) the fatty acid concentration dropped below the critical micelle concentration (Mukerjee and Mysels, 1971). Therefore, it appeared that trypsin inhibition observed in this study was attributed to direct binding of the ionized fatty acid monomer to trypsin rather than to a detergent effect of the inhibitory compounds.

Accordingly, the more ionized a fatty acid, the more ions would be available for binding the enzyme and the stronger would the inhibition be. This was actually observed when linoleate was tested for trypsin inhibition at various pH levels (Figure 3).

The protective effect of Ca^{2+} (Figure 4) might be due to its capability of binding to the ionized fatty acid as well as to trypsin itself. Binding of Ca^{2+} to the ionized fatty acid results in reduction in both solubility and ionization of the latter, while interaction of Ca^{2+} with trypsin results in a conformational change of the enzyme (Green and Neurath, 1953). In both cases, the capacity of binding between trypsin and the ionized fatty acid monomer would be altered. The fact that addition of Ca^{2+} after preincubation of inhibitor with enzyme provided no protective effect indicates that, once the binding of ionized inhibitory compound to enzyme is accomplished, Ca^{2+} ions can no longer interfere with the binding process.

The time and temperature dependency of the inhibition might suggest that there is an energy barrier for binding, which is overcome by increasing the temperature and time of interaction between enzyme and inhibitor.

The binding process may involve both hydrophilic and hydrophobic interactions between the inhibitor and the enzyme, which in turn affect the secondary and tertiary structure of the enzyme. The increased trypsin inhibition with increase of carbon chain length of fatty acids (Figure 6) may be due to increasing hydrophobic interaction between inhibitor and enzyme.

The higher trypsin inhibition by stearoyl-CoA as compared with its corresponding fatty acid can also be explained by the monomer binding hypothesis, since the thioester has higher solubility than its corresponding fatty acid.

Finally, while trypsin inhibition by free fatty acids follows the general characteristics described earlier for enzyme inhibition by fatty acids, the inhibition as a function of the ionized state of inhibitory compounds, as well as the protective effects of Ca^{2+} on trypsin, observed in this study suggests that these two additional features may apply to the inhibition of other enzymes by fatty acids. The lack of selective inhibition provides a basis for some previous investigators to suggest that effects of long-chain fatty acids and their acyl-CoA esters in vitro on enzyme action may be insignificant for metabolic regulation in vivo (Taketa and Pogell, 1986; Pande and Mead, 1968; Parvin and Dakshinamurti, 1970). The two newly found features provide another basis to support such a proposal; in the gut, salts can readily abolish the inhibition.

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Registry No. Ca, 7440-70-2; trypsin, 9002-07-7; potassium butanoate, 589-39-9; potassium hexanoate, 19455-00-6; potassium octanoate, 764-71-6; potassium caprate, 13040-18-1; potassium laurate, 10124-65-9; potassium myristate, 13429-27-1; potassium palmitate, 2624-31-9; potassium stearate, 593-29-3; potassium arachidate, 18080-76-7; potassium oleate, 143-18-0; potassium linoleate, 3414-89-9; potassium linolenate, 38660-45-6; stearoyl-CoA, 362-66-3.