

Inhibition of Milk Kinin Activity by Phenolic Antioxidants

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Abstract

The smooth muscle contracting ability of a kinin released by trypsin from a kininogenic source in milk is inhibited by the presence of water-soluble antioxidants of the phenolic type. The manner by which these antioxidants inhibit this physiological response to milk kinin is similar to that observed for bradykinin. The initial suppression of the contractile response of the isolated guinea pig ileum by gallic acid and members of a homologous series of gallate esters increased almost directly with increasing chain length of the acid substitution group. Butylated hydroxyanisole was equally effective in suppressing milk kinin activity. Plots of reciprocal of gut response versus the reciprocal of kinin concentration for different concentrations of these food grade antioxidants indicated they might act as competitive inhibitors. The suppressive effects of the antioxidants were a function of purity of the kininogen preparation.

Introduction

Bovine plasma kininogens have been prepared by Habermann et al. (5) and Suzuki et al. (9). A physiologically active peptide released from bovine plasma by the action of trypsin has been identified as bradykinin (2).

In 1959 Guth described the presence of a kininogen in bovine colostrum (4). His studies indicated that the kininogen content of milk decreased to zero shortly after calving. However, Leach et al. (6) found that small amounts of kininogen are in all mixed herd milks used for human consumption.

Since the relationship between blood kininogen and bradykinin and the materials in milk is unknown, we have partially purified milk kininogen for further study. During our research we noted that some common food grade

antioxidants will interfere with the biological assay used to determine the presence of the kininogen. We had previously shown that these same antioxidants also suppress ileum response to bradykinin in an apparently complex manner (7, 8).

The purpose of this paper was to investigate the inhibitory action of these antioxidants on smooth muscle contraction effected by milk kinin to make further comparisons with bradykinin possible.

Materials and Methods¹

Preparation of kininogen. Crude kininogen was prepared from acid whey by treatment with trichloroacetic acid to remove β -lactoglobulin by the method of Fox et al. (3) and by salt fractionation with ammonium sulfate to remove α -lactalbumin by the method of Aschaffenburg (1). Further purification was effected by fractionation on polyacrylamide gel (PAG) using a Prep-Disc electrophoresis apparatus (Canal Industrial Co.). Polyacrylamide gel patterns of the purified protein were made with a Canalco analytical electrophoresis apparatus according to the manufacturer's directions with a Standard Separating Gel, pH 9.5.

Assuming a molecular weight and biological activity similar to the kininogen isolated from blood plasma, the concentration of kininogen and milk kinin in the test samples was determined by the guinea pig ileum's response to synthetic bradykinin (BRS 640, Sandoz Pharmaceutical Co.).

Biological assay. Assays were conducted with a 2 to 3 cm section of the terminal segment of guinea pig ileum which was suspended in a 5-ml perfusion bath held at 37 C. Changes in length of the ileum were recorded with a standard kymograph. The gut was equilibrated for 1 hr in a carrier solution of Tyrode's buffer containing 2 mg/liter of atropine and 40 μ g/liter pyribenzamine. Stock solutions of kininogen were prepared in Tyrode's buffer. Control responses to milk kinin were determined by injection of a .2-ml aliquot of stock solution after a 10 min incubation with .1 mg trypsin (2 \times crystalline, Nutritional Biochemical Corp.) per milliliter of kininogen stock solution into the reaction chamber after

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¹Trade names are mentioned for identification, implying no endorsement.

buffer flow had been interrupted. After a 30-sec exposure of the smooth muscle to the kinin, the cell was flushed by buffer at 15 ml/min.

Assays in the presence of inhibitors were conducted under the same conditions. Stock solutions of gallic acid (Fisher Chemical Co.), butylated hydroxyanisole (Nutritional Biochemical Corp.) and the gallate esters, methyl, ethyl, N-propyl, iso-propyl, N-butyl, N-amyl, and N-hexyl, prepared according to the procedure of Russell and Tebbens (11), were made up in distilled water. All the esters except hexyl were prepared from chromatographically pure alcohols and melted at values reported in the literature. The esters migrated as a single spot when analyzed by thin layer chromatography. Suitable concentrations of these antioxidants were mixed with the incubated material immediately before injection into the bath. Decreases in peak heights in the presence of the antioxidants were used to evaluate their inhibitory action.

The mechanism of this action and the inhibitory potency of the antioxidants are ascertained from drug-dose response studies as described by Roche e Silva (10) whereby the reciprocals of effects ($1/Y$) where Y is the peak height in millimeters as measured on the kymograph are plotted versus reciprocals of doses ($1/X$) where X is the concentration of

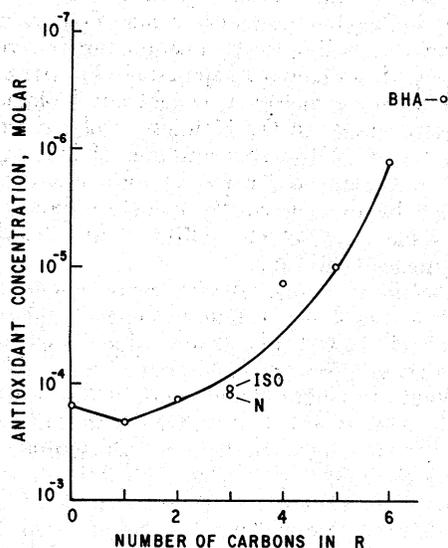


FIG. 1. Minimum concentration of gallic acid and its esters required to detectably inhibit ileum response to milk kinin doses equivalent to .04 μ g/ml bradykinin. R = group derived from the alcohol in the ester $C_6H_5O_2COOR$.

TABLE 1. Increase in molar concentrations of antioxidants required to produce a 25 to 35% reduction in control responses to milk kinin doses equivalent to .04 μ g/ml bradykinin.

Antioxidant	Increase %
Gallic acid	170
Methyl gallate	110
Ethyl gallate	200
N-Propyl gallate	250
Isopropyl gallate	330
Butyl gallate	200
Amyl gallate	490
Hexyl gallate	2,500
Butylated hydroxyanisole	1,000

kinin. From the ratio of the slopes of the double-reciprocal plots and the concentration of inhibitor present, the inhibitory strength of the antioxidants can be expressed as the pKi value.

All concentrations are presented as concentrations in the reaction bath.

Results

The thresholds at which gallic acid and its six homologous esters begin to produce an inhibition of ileum response to milk kinin released from a crude kininogen preparation of approximately 5% purity is presented in Figure 1. Butylated hydroxyanisole (BHA) is shown for comparison. Very little difference is noted in the repressive strength of the isomeric forms of propyl gallate.

The amounts by which the molar antioxidant concentrations must be increased to effect a 25 to 35% reduction of control responses to milk kinin doses from the preparation of 5% purity are presented in Table 1. Larger increases in amyl and hexyl derivatives and BHA are required to produce the same suppression as the shorter chained esters. Longer flushings were necessary to return the ileum to normal kinin sensitivity after doses of the butyl, amyl, and hexyl esters and BHA. Control responses were irreversibly decreased after repeated doses of these compounds.

TABLE 2. Concentration of N-propyl gallate required to initiate suppression of response to kinin doses equivalent to .04 μ g/ml bradykinin.

Kinin source	Concentration (molar)
5% Pure preparation	1.8×10^{-4}
80% Pure preparation	4.0×10^{-6}
Bradykinin	2.0×10^{-7}

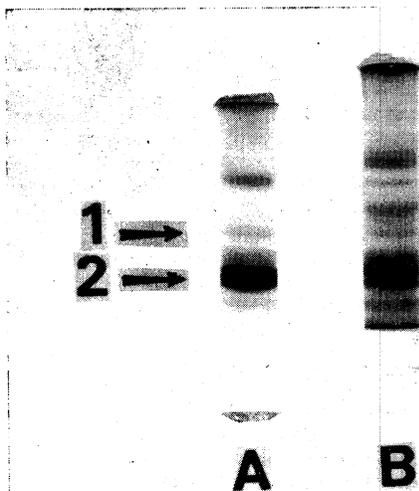


FIG. 2. Polyacrylamide gel electrophoretic patterns of the (A) 80% and (B) 5% pure preparations of kininogen. Component 1: Kininogen. Component 2: Bovine serum albumin.

The effectiveness of N-propyl gallate in suppressing the action of kinin released from a kininogen preparation of 80% purity is increased as is shown in Table 2. The antioxidant has an even greater effectiveness against pure bradykinin.

The electrophoresis patterns in PAG of these preparations are in Figure 2.

To establish the nature of the kinin inhibition of the antioxidants, double reciprocal plots were established for four of these compounds with the 80% pure preparation as the milk kinin source. An example of these plots is in Figure 3. The relatively straight lines passing through a common point on the Y axis are

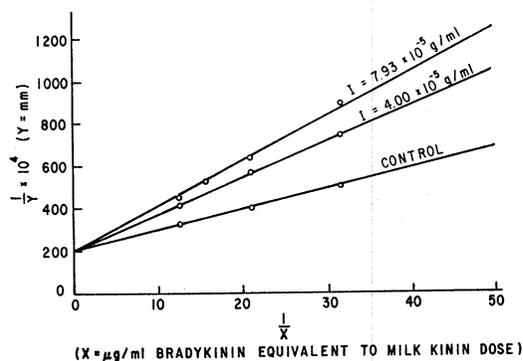


FIG. 3. Drug-dose (X) - response (Y) plot for ethyl gallate. I = gallate concentration.

TABLE 3. pKi Values (w/v) of antioxidants.

Antioxidant	pKi (w/v)
Ethyl gallate	4.29
N-Propyl gallate	5.25
Hexyl gallate	5.85
Butylated hydroxyanisole	5.54

typical of those obtained when a competitive inhibitor is influencing a biological system. Similar curves were obtained for N-propyl and hexyl gallate. Butylated hydroxyanisole produced a deviation from linearity with low kinin doses.

pKi Values calculated from the graphical data are in Table 3. The values for BHA are derived from those portions of the curves describing straight lines. These values indicate that the compounds tested are relatively weak inhibitors of milk kinin activity.

Discussion

Milk contains a kininogen which gives rise to a kinin capable of inducing smooth muscle contraction. This physiological response to the presence of milk kinin is suppressed by the phenolic antioxidants tested in a complex manner similar to the inhibition of bradykinin induced ileum contraction (7, 8). The threshold concentrations of antioxidants required to detectably inhibit ileum response to either kinin appear to be almost directly related to the chain length of the acid substitution group with BHA being the most effective agent tested. Gallic acid presents the only deviation from this pattern. Larger changes in the concentration of the longer chained esters and BHA are needed to produce more significant suppression of response.

The amount of antioxidant required to suppress action on contractile response to milk kinin released from the 80% pure kininogen preparation is closer to that required to produce the same effect on response to bradykinin than is the concentration of antioxidant required to suppress a response to kinin released from the 5% pure preparation. This is probably due to antioxidant binding by milk proteins in the preparations.

The possibly competitive nature of the inhibition and only partial reversibility of response to control doses of milk kinin are similar to results obtained with pure bradykinin. The nature of milk kinin inhibition is not typically competitive in that relatively large increases in antioxidant are required to change

the slopes of the lines of the double reciprocal plots. As we have previously reported, these antioxidants do not inhibit histamine incited contraction, and it is inferred that the antioxidants are bound to specific kinin acceptor sites in or on the muscle cells.

Although these antioxidants inhibit ileum response to milk kinin similar to that observed for bradykinin, the proof of exact similarity awaits further purification of the milk kininogen.

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