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Resistance of Cranberry Bean (*Phaseolus vulgaris*) α-Amylase Inhibitor to Intraluminal Digestion and its Movement along Rat Gastrointestine: Further Investigation using a Radioactive Probe and Specific Antiserum

Makoto Kotaru

Department of Home Economics, Koka Women's Junior College, Kyoto 615, Japan

Kimikazu Iwami, Hung-Yu Yeh & Fumio Ibuki

Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto Prefectural University, Kyoto 606, Japan

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ABSTRACT

*Cranberry bean (*Phaseolus vulgaris*) α-amylase inhibitor (CBAI) was treated with [^{14}C]HCHO and $NaBH_4$ to modify the lysyl ε-amino group. [^{14}C] Labelled inhibitor thus obtained was used as a radioactive probe. The reductive methylation of CBAI caused neither a significant change in its susceptibility to digestive enzymes nor in its immuno-reactivity with rabbit antiserum. Consequently, gastric emptying and intestinal transit of the inhibitor was represented by radioactivity measurement and simultaneously complemented by quantification due to single radial immuno diffusion. As a result, the bulk of the inhibitor was found not to undergo digestion and absorption but to pass through the small intestine without much loss of antigenicity and radioactivity.*

INTRODUCTION

Proteinaceous inhibitors highly specific for mammalian α-amylase have been found in a variety of cereals, legumes and tubers (Buonocore & Silano,

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1986). Much interest has arisen in their use for therapy of *diabetes mellitus* or obesity, because their oral administration is expected to prevent starch digestion and thereby to cause a great calorie cut. In this regard, Plus and Keup (1973) first reported that wheat α-amylase inhibitor had an inhibitory effect on amylolysis in the digestive tract of rat, dog and human. Layer *et al.* (1986) also demonstrated that white kidney bean α-amylase inhibitor was effective in lowering postprandial glycemia in human subjects. Similar effects in rats were obtained with α-amylase inhibitor from black kidney beans (Menezes & Lajolo, 1987) and from cranberry bean (Kotaru *et al.*, 1989*b*). However, the previous observations gave apparent support to the possibility of practical application of the inhibitor, but little information on how it escapes attack by proteases and acts on α-amylase in the lumen. With respect to *in vitro* digestibility of cranberry bean α-amylase inhibitor (CBAI), we have found that CBAI is quite resistant to pepsin and trypsin digestions, but seemingly susceptible to chymotrypsin digestion (Kotaru *et al.*, 1989*a*).

The present paper deals with the results of an investigation on the intraluminal behavior of CBAI containing [^{14}C]methylated CBAI, after its gastric intubation, from the antigenic and radioactive viewpoints.

MATERIALS AND METHODS

Materials

CBAI was prepared as previously described (Kotaru *et al.*, 1987*a*). Porcine α-amylase, bovine trypsin, bovine chymotrypsin, porcine pepsin and cholecystokinin octapeptide (CCK-8) were purchased from Sigma Chemical Co., St Louis. [^{14}C]Formaldehyde (10 mCi/mol), [^{14}C]toluene ($4{\cdot}7 \times 10^5$ dpm/ml) and Protosol (a tissue solubilizer) were products of New England Nuclear, Boston. Clearsol for liquid scintillation and 3,5-dinitrosalicylic acid for amylase assay were from Naclai Tesque Inc., Kyoto. All other chemicals were of analytical grade, commercially available.

Methods

Labelling of CBAI with radioisotope

Reductive methylation of CBAI with [^{14}C]HCHO and $NaBH_4$ was carried out according to Rice and Means (1971); namely, CBAI (9 mg) was dissolved in 2 ml of 0·2M (pH 9) borate buffer, to which was added 10 μl of 40 mM [^{14}C]HCHO (4·6 mCi/mol) and 50 μl of 15 mM $NaBH_4$ five times at 30 s intervals. The reaction mixture was allowed to stand for 6 h on ice-cold

water and dialyzed several times against 500 ml of distilled water. These operations were repeated five times and the dialyzates were combined, followed by lyophilization.

Animal experiment
Male Wistar rats weighing about 160 g, that had been fasted overnight, were given, by gastric intubation, 1 ml of a saline solution containing 2 mg of [^{14}C]-labelled CBAI and 8 mg of native CBAI, and at the same time, 100 μl of a saline solution containing 1 μg of CCK-8 was injected into the tail vein so as to stimulate pancreatic exocytosis. The rats were sacrificed at stated intervals to excise the gastrointestine, which was divided into stomach, three equal small bowel segments (proximal, middle and distal parts) and caecum. Their corresponding intraluminal contents and washings were combined, individually lyophilized and stored in the refrigerator until use. Several rats receiving 1 ml of saline containing no CBAI were used for reference.

Enzyme assays
The activities of α-amylase and its inhibitor were estimated using 3,5-dinitrosalicylic acid as previously described (Kotaru *et al.*, 1987*b*). The trypsin and chymotrypsin activities were colorimetrically determined using *p*-nitroanilides of α-*N*-benzoyl-DL-arginine and *N*-benzoyl-L-tyrosine, respectively, as substrates for the sake of convenience. Estimation of the pepsin activity was due to UV measurement of digested casein.

Immunoassays
Double immunodiffusion was done in the usual way (Ouchterlony, 1958, 1962); the centre and peripheral wells in 2 mm thick agarose gel were charged with the same rabbit anti-CBAI serum as previously made (Kotaru *et al.*, 1991) and HCHO-treated and untreated CBAI samples, respectively. The gel after a day's diffusion was fully rinsed in phosphate buffer and stained with Coomassie brilliant blue. Quantification of the intraluminal CBAI content in each segment was due to single radial diffusion (Mancini *et al.*, 1965).

Radioactivity measurement
An aliquot of the intraluminal content (0·1 ml) was decolored by treatment at 50°C for 30 min with an equal volume of hydrogen peroxide, and then dissolved in 0·3 ml of Protosol. After addition of 10 ml of Clearsol per vial, the radioactivity was measured with a Packard-Tricarb liquid scintillation counter. Corrections on quenching due to inadequate decolorization were made using [^{14}C]toluene as a standard.

Statistical analysis

This was evaluated on the basis of Student's *t*-test to find whether there is a significant difference between two means when the *F*-test was significant ($P < 0{\cdot}05$) as a result of variance analysis.

RESULTS AND DISCUSSION

Justification of CBAI modification with [^{14}C]HCHO

CBAI was treated with [^{14}C]HCHO and $NaBH_4$ in order to obtain a radioactive probe. The efficiency of [^{14}C]methylation was, however, far less than expected. CBAI with molecular weight of 45 000 contains four lysine residues per mole (Kotaru *et al.*, 1987*a*), so that at least 22 nmoles of [^{14}C]HCHO per mg of CBAI can be linked to the ε-amino group of lysine, corresponding to 0·1 μCi ($2{\cdot}2 \times 10^5$ dpm). Actual counting of [^{14}C]HCHO-treated CBAI was approximately $1{\cdot}5 \times 10^4$ dpm per mg of CBAI, implying that only partial lysine residues underwent chemical modification. Even if this were so, the treatment of CBAI with HCHO and $NaBH_4$ may possibly cause a change in its antigenicity. Figure 1 shows the result of double immunodiffusion in agarose gel of the antiserum against CBAI samples with and without reductive methylation. Three precipitin lines were fused together with each other, and no spur was observed at each fusion point. For comparison, CBAI was treated with a large excess amount of cold HCHO.

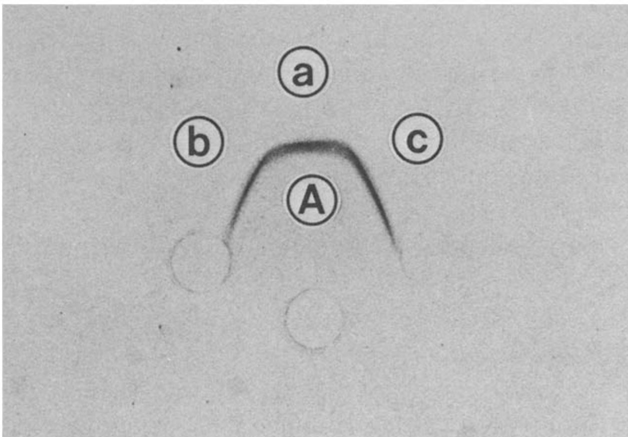

Fig. 1. Double immunodiffusion between rabbit anti-CBAI serum and CBAI with or without HCHO treatment. A, anti-CBAI serum; a, native CBAI; b, [^{14}C]HCHO-treated CBAI; c, CBAI treated with excess HCHO. A single fused precipitin line in agarose gel was made more visible by staining the plate with Coomassie brilliant blue.

As a matter of course, the efficiency of CBAI methylation ought to have been markedly enhanced. Double immunodiffusion of the specific antiserum against more modified CBAI resulted in a single precipitin line without spur.

Subsequently, the process of reductive methylation was examined to see if it affects *in vitro* digestibility of CBAI. Figure 2 illustrates the result of CBAI digestion by chymotrypsin. Both HCHO-treated and untreated CBAI samples were unsusceptible to digestion by pepsin or trypsin (data not shown). On the contrary, the inhibitor activities of CBAI samples with and without chemical modification decreased gradually with prolonged chymotrypsin digestion, and largely disappeared at the first 30 min in the presence of chymotrypsin at a 10-fold molar concentration as much as CBAI. There was no difference in the loss of inhibitor activity between these modified and native samples. The decay curve for antigenicity sloped more gently than that for inhibitor activity. Even in this instance, differences between the two CBAI samples would be impossible. Such is the case in practice. The use of [^{14}C]HCHO-treated CBAI as a radioactive probe can be regarded as reasonable, because the reductive methylation of CBAI neither affected its inhibitor activity (Kotaru *et al.*, 1989*a*) nor its antigenicity.

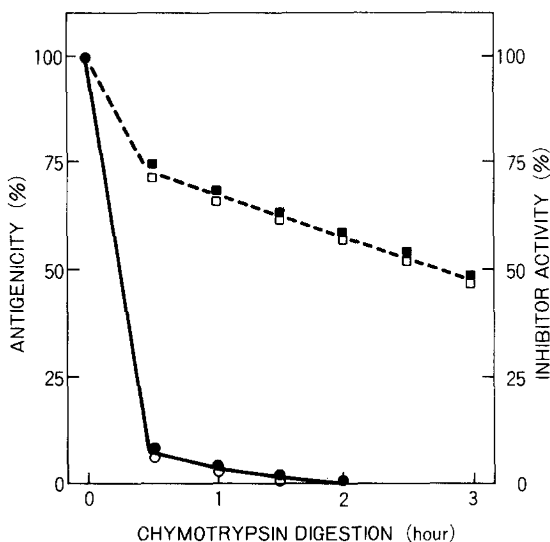

Fig. 2. Effect of HCHO treatment on susceptibility of CBAI to chymotrypsin digestion. Reaction mixture at 37°C contained $2{\cdot}2 \times 10^{-5}$M HCHO-treated or untreated CBAI and $2{\cdot}2 \times 10^{-4}$M bovine chymotrypsin. Aliquots were withdrawn at stated intervals, followed by inhibitor activity and antigenicity measurements. The antigenicity was estimated as the area of the hole by single radial immunodiffusion. Inhibitor activity: ○, HCHO-treated CBAI; ●, native CBAI. Antigenicity: □, HCHO-treated CBAI; ■, native CBAI.

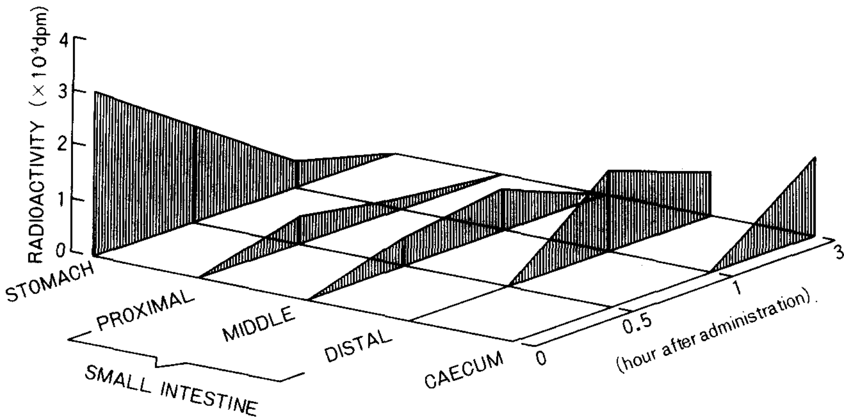

Fig. 3. Distribution of radioactive CBAI in gastrointestine at stated intervals after its administration. A mixture of cold CBAI and [^{14}C]labelled CBAI was administered by gastric intubation to rats, which received an intravenous injection CCK-8 at the same time. At each prescribed time, four rats were sacrificed and the digestive tract was excised to measure the residual radioactivity in its regions.

Intraluminal movement of CBAI along gastrointestine

Figure 3 depicts the intraluminal inhibitor distribution in rat gastrointestine, which was represented by the proportion of radioactive CBAI in the respective segments at stated intervals after a dosage of 10 mg of CBAI containing 0·007 μCi of [^{14}C]methylated one. CBAI was thoroughly emptied from the stomach in 3 h. In this regard, gastric emptying seemed to be earlier than that ordinarily observed for rats given a solid chow. This is because CBAI was administered in dilute solution and moderate quantity. The radioactivity levels in the proximal and middle thirds of small intestine were relatively low at the first two time points (0·5 h and 1 h) after the CBAI administration, and were almost missing in 3 h. This fact suggests that chyme would have smoothly passed through these intralumens. The intraluminal radioactivity became maximal in 1 h in the distal third, and was still present in 3 h, as it was in 3 h in the caecum. Since the standard deviation from the average recovery ranged from 12·5 to 36% ($n=4$), the amount of administered [^{14}C]CBAI can be regarded as almost quantitatively recovered at each measuring time.

Figure 4 summarizes the results of single radial immunodiffusion of CBAI remaining in the respective segments at indicated times. The residual CBAI levels estimated from this viewpoint were seemingly higher in the proximal and middle thirds and lower in caecum as compared with those simultaneously done on the basis of radioactivity measurement. Taking into account the experimental error (less than 10%) and standard deviation

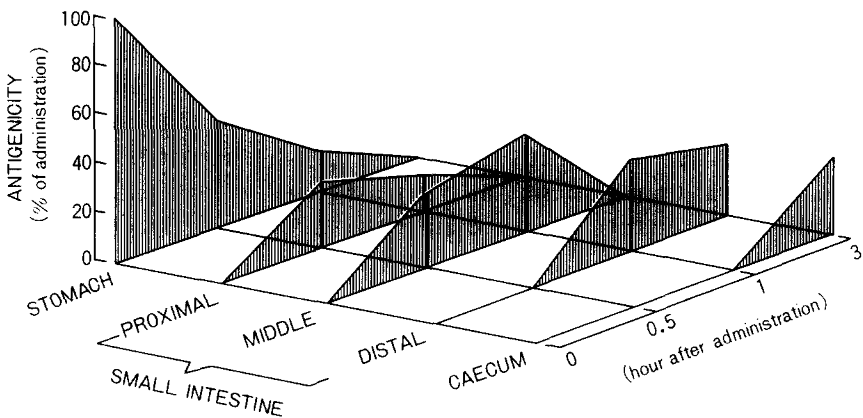

Fig. 4. Distribution of immuno-reactive CBAI in gastrointestine at stated intervals after its administration. The animals used in this assay were the same as in Fig. 3. The amount of immuno-reactive CBAI, i.e. the antigenicity level, remaining in each region at a prescribed time was estimated as above, and expressed in percentage of that in the stomach at zero time.

(ranged from 8 to 25%), however, a similar relation may safely be said to hold (on the whole) between the intraluminal CBAI movements judged by antigenicity and radioactivity.

CBAI is not affected by the action of pepsin itself, although its inhibitor activity is a little lowered when exposed to acidic pH (below pH 2) (Kotaru *et al.*, 1987*b*). No significant loss was observed in the antigenicity of CBAI during its stay in the stomach. On the other hand, it was assumed from the *in vitro* digestion experiment that CBAI would not be sensitive to trypsin digestion but to chymotrypsin digestion (Kotaru *et al.*, 1989*a*). Even if its proteolysis should take place to a small extent in the duodenojejunum, the amount of CBAI recovered from the small intestine, which was obtained by radioactivity and antigenicity measurements, was almost equal to that dispatched from the stomach at early stages after administration. In addition to such a quantitative recovery, the agreement between both radioactivity and antigenicity determinations at these measuring times suggests that CBAI would have been saved from intraluminal digestion. Interestingly, the average recovery of CBAI from the ileocaecum 3 h after its administration seemed to be less in antigenicity than in radioactivity. This difference can be fairly interpreted by considering that a partial cleavage of the peptide bond or a structural change leading to a decrease in antigenicity takes precedence over intestinal absorption leading to a decrease in radioactivity.

The amount of CBAI administration used in this experiment was too little to stimulate pancreatic exocytosis. For the purpose of raising secretion of the pancreatic juice, CCK-8 (1 μg) was injected into the tail vein. Prior to its

injection, rats were forcibly given a saline solution containing 10 mg of CBAI. Twenty minutes later, the output of digestive enzymes began to increase and continued for a while. About that time, one-third to half of administered CBAI had been transferred from the stomach to the intestine, in which CBAI was in contact with secreted digestive enzymes. Practically speaking, intraluminal α-amylase activity was significantly low in duodenojejunum relative to the reference without CBAI loading as previously demonstrated (Kotaru *et al.*, 1989*b*). The results of radioactivity and antigenicity measurements implied that CBAI, rid of digestion and absorption, was transferred to the ileocaecum to some extent. Even so, it is doubtful whether the inhibitor activity of CBAI remains intact there. When considered with a somewhat poor recovery of immunoreactive CBAI from there, it is more likely that CBAI has been much deprived of its inhibitor activity. When the use of too much protease in the *in vitro* model experiment is taken into consideration, it is highly probable that CBAI escapes intraluminal digestion beyond expectations. If it were so, CBAI would be useful as a non-poisonous pharmaceutical for improvement in hyperglycemia.

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