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Isolation and partial characterization of pigeon pea protease inhibitor: its effect on the genotoxic action of aflatoxin B_1

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Abstract—Protease inhibitor was isolated and purified from pigeon pea *Cajanus capan*. By using gel filtration analysis the inhibitor was found to have an M_r of 18,200. It inhibits trypsin competitively with a specific inhibitor constant K_i of 1.53×10^{-7} M. The purified inhibitor produced a marked reduction in aflatoxin B_1-induced β-galactosidase activity in *Escherichia coli* PQ37. This reduction is independent of whether the protease inhibitor was added to the reaction medium prior to or after aflatoxin B_1 activation. The observed reduction may therefore be a result of the inhibitor's activity on the RecA protease produced in response to aflatoxin B_1-induced DNA damage in the bacteria.

Protease inhibitors are a ubiquitous group of proteins found in microorganisms, plants and animals [1]. These proteins have diverse biochemical functions, some of which include intracellular catabolism of peptides and proteins [2], inhibition of intraerythrocytic development of *Plasmodium falciparum* [3], suppression of *in vitro* and *in vivo* replication of retroviruses [4] and inhibition of growth of transformed cells [5, 6]. Studies have been intensified on the mechanism of anticarcinogenicity exhibited by these inhibitors [7–9]. Increased exposure to dietary protease inhibitors has been shown to protect against some chemically induced animal tumours [10, 11] and it has been suggested that protection against cancer may be achieved through this avenue [12].

In an attempt to acquire more information on the mechanism of anticarcinogenesis by protease inhibitors, we describe here the influence of protease inhibitor isolated from pigeon pea on the genotoxicity of aflatoxin B_1 (AFB_1*), a potent animal hepatocarcinogen [13] in *Escherichia coli* PQ 37.

Materials and Methods

The pigeon pea was obtained from the Agricultural farm of the University of Ibadan, Ibadan, Nigeria.

All the chemicals used were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Isolation and purification of protease inhibitor from pigeon pea. This was carried out by a modified method of Sohonie and Ambe [14]. Finely ground peas (200 g) were defatted with 1 L 80% ethanol for 30 min. The mixture was filtered and the meal extracted with 1 L 0.25 M H_2SO_4 at 30° for 1 hr. The suspension was centrifuged for 30 min at 1200 *g* and the supernatant was half-saturated with ammonium sulphate. The precipitate was then taken up with 100 mL of 2.5% trichloroacetic acid and heated in a boiling water bath for 5 min. The suspension obtained was chilled immediately in ice and filtered. The filtrate was half-saturated again with ammonium sulphate and centrifuged. The residue was dissolved in 20 mL water and dialysed in water. The content of the dialysis bag was centrifuged and the supernatant precipitated by four volumes of cold acetone. The acetone precipitate was separated and dissolved in water. From the inhibitor specific activity of the crude extract, the peas contain 0.31 g of inhibitor per 200 g.

The extract was purified by gel filtration on Sephadex G-75. Trypsin inhibitor activity in 2 mL fractions of the effluent was measured by the casein digestion method of Kunitz [15], while their protein content was detected by measuring the optical density at 280 nm. Fractions containing the highest concentrations of inhibitor were pooled together, concentrated and stored frozen for subsequent tests.

Effect of pigeon pea inhibitor on AFB_1 induced genotoxicity. This was studied by following the SOS chromotest procedure of Quillardet and Hofnung [16]. The bacterial strain *E. coli* PQ37 was used after it had been checked for the presence of the *rfa* and *uvr*A mutations using deoxycholate and UV sensitivity tests, respectively [17]. The culture media and other solutions were prepared as reported by Quillardet and Hofnung [16]. Samples of 0.3 mL of an exponentially growing culture of the tester bacteria, freshly diluted in the S9 activation mixture, were added into tubes containing graded concentrations of AFB_1 in 10 μL dimethyl sulphoxide (DMSO). The same volume of bacteria solution was added into tubes containing a mixture of graded concentrations of the purified protease inhibitor and the maximal inducing concentration of AFB_1 in a final volume of 10 μL. In the third assay, the bacteria samples were added into tubes containing the maximal inducing concentration of AFB_1 followed by the addition of the graded concentrations of the protease inhibitor, the final volume of the test compounds being 10 μL. The mixtures were then incubated for 2 hr at 37° after which β-galactosidase activity was assayed as described in Ref. 16 and the enzyme units calculated [18]. The assays were performed in triplicate.

Results and Discussion

The M_r of the isolated pigeon pea inhibitor was estimated to be 18,200 on gel filtration analysis. This falls within the M_r range of 8000–25,000 which has been reported for various protease inhibitors. The activity of the inhibitor on trypsin gave a linear function (Fig. 1), this behaviour has been shown to be common to a majority of isolated protease inhibitors [19]. It is apparent from the Lineweaver–Burk plot of enzyme activity in Fig. 2 that the inhibitor interacts with trypsin, acting as a competitive inhibitor with K_i of 1.53×10^{-7} M.

In the SOS chromotest, it was observed that 8 μg AFB_1 per assay induced β-galactosidase maximally in the tester bacteria *E. coli* PQ37 (Fig. 3). The enzyme induction curve ascends gradually until the maximum value is reached at the toxic level after which the induction falls. AFB_1 metabolites produce a deleterious effect in the bacteria at the toxic level leading to inhibition of enzyme synthesis. The presence of the pigeon pea trypsin inhibitor in the assay medium led to a marked decrease in the degree of β-galactosidase induced by the maximal inducing concentration of AFB_1 (Fig. 3). The effect of the inhibitor

* Abbreviations: AFB_1, aflatoxin B_1; DMSO, dimethylsulphoxide.

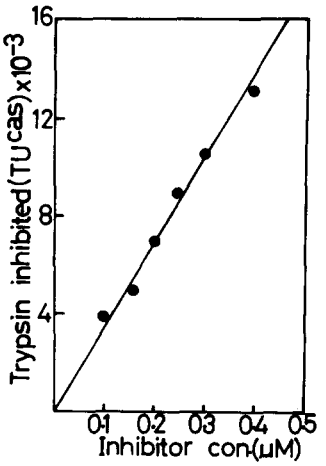

Fig. 1. Inhibition of trypsin by pigeon pea protease inhibitor. TU^{cas}, the amount of trypsin inhibited per unit weight of protease inhibitor expressed in tryptic units.

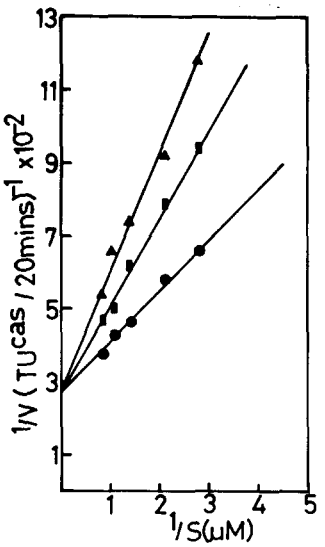

Fig. 2. Lineweaver–Burk plot of trypsin activity in the absence (●) and presence of pigeon pea protease inhibitor (■ 0.11 μM; ▲ 0.22 μM). TU^{cas}, as in legend to Fig. 1.

is independent of whether the inhibitor was added before or after AFB_1 activation to the 2,3-epoxide. This suggests that the pigeon pea inhibitor does not interfere with AFB_1 activation and therefore the initiation of the genotoxic damage.

The tester bacteria *E. coli* PQ37 has an operon fusion (*sfi*A: *lac*Z) which places the structural gene for β-galactosidase under the control of the *sfi*A gene which codes for an SOS function involved in the inhibition of cell division [20]. Expression of the operon fusion is normally repressed by the Lex A protein. AFB_1, like other

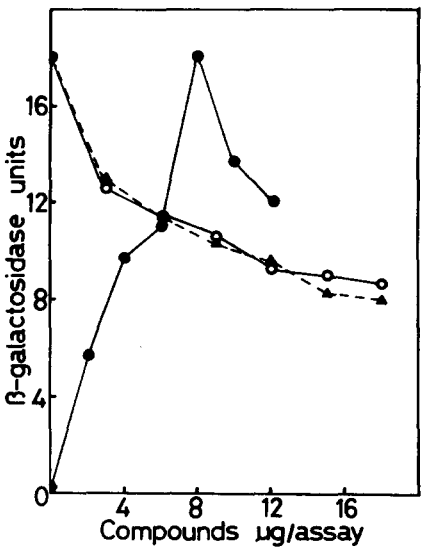

Fig. 3. Effect of pigeon pea protease inhibitor on β-galactosidase induction by AFB_1. AFB_1 alone at various concentrations in 10 μL DMSO (●); ABF_1 (8 μg) in the presence of graded concentrations of the protease inhibitor added before AFB_1 activation (▲) and after AFB_1 activation (○) in a final volume of 10 μL.

genotoxins, induces damage in the bacteria via the epoxide reacting with the DNA and consequently converting the Rec A protein produced in response to SOS function induction to a Rec A protease which cleaves the Lex A protein leading to the derepression and expression of the *sfi*A and *lac*Z genes. It is therefore probable that the mechanism of action of the pigeon pea trypsin inhibitor involves the inhibitor reacting with Rec A protease and thus inhibiting its activity on the Lex A protein with consequent inexpression of the operon and inhibition of β-galactosidase synthesis.

Proteases have been suspected to play an essential role in the initiation and maintenance of carcinogenicity [21] and it has been suggested that inhibition of growth of transformed cells by protease inhibitors may be due to inhibition of a protease-like activity required for unrestrained growth [5]. Observations made in this study with the pigeon pea inhibitor and AFB_1 seem to agree with these suggestions. It is becoming obvious that protease inhibitors do in fact possess anticarcinogenic/antimutagenic properties, though the understanding of their actual mechanism of action is still underway. It is, however, envisaged that the target enzymes of many protease inhibitors would be identified in the near future and this may be the key to a better understanding of their mechanism of action.

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Stimulation of dopamine D_1 receptors by the D_2 agonist CV 205-502 in bovine retina homogenates

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CV 205-502, an octahydrobenzo(*g*)quinoline, combines the essential dopaminomimetic pharmacophore of apomorphine with the long duration of action and the good oral activity of the ergolines [1]. So far it has been used on the one hand as a high-affinity selective dopamine (DA*) D_2 receptor agonist ligand in autoradiographic studies [2, 3], and on the other hand as a prolactin inhibitor and an alternative to bromocriptine in the treatment of pituitary prolactinomas and of hyperprolactinaemia [4, 5]. In the autoradiographic studies, [^{3}H]CV 205-502 binding was displaced with high affinity by DA D_2 receptor agonists or antagonists such as apomorphine, spiroperidol, *S*(−)-sulpiride and (+)-butaclamol, but not by the inactive enantiomer (−)-butaclamol; the DA D_1 receptor agonist SKF 38393 and the D_1 antagonist *R*(+)-SCH 23390 were also ineffective [3]. However, binding studies in the rat striatum have shown that [^{3}H]CV 205-502 is very weakly displaced by SCH 23390, with an IC_{50} of 1.98 ± 0.7 μM [2]. To our knowledge, no direct biochemical evidence has so far been provided to show that CV 205-502 is able to stimulate DA D_1 receptors positively linked to adenylate cyclase [6] and to concomitantly generate dose-dependent increases in cAMP concentrations.

The retina embodies many of the features that are distinctive of the central nervous system in general and does so within a structure that is relatively simple. Its easy isolation and remarkable survival as well as the identification of DA as a retinal neurotransmitter make it a useful model for the biochemical characterization of the DA-sensitive adenylate cyclase (Refs 7; 8 for a review and references therein). Bovine retina homogenates were thus used in this investigation to test whether CV 205-502 could possibly induce the formation of cAMP through the stimulation of DA D_1 receptors.

* Abbreviations: DA, dopamine; EtOH, ethanol.