Isolation of Unguilin, a Cyclophilin-Like Protein with Anti-Mitogenic, Antiviral, and Antifungal Activities, from Black-Eyed Pea

X. Y. Ye and T. B. Ng

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A protein designated unguilin was isolated from seeds of the black-eyed pea (Vigna unguiculata). It possesses a molecular weight of 18 kDa and an N-terminal sequence resembling that of cyclophilins and the cyclophilin-like antifungal protein from mung beans, and was adsorbed on Affi-gel blue gel and CM-Sepharose. Unguilin exerted an antifungal effect toward fungi including Coprinus comatus, Mycosphaerella arachidicola, and Botrytis cinerea. In addition, unguilin was capable of inhibiting human immunodeficiency virus-1 reverse transcriptase and the glycohydrolases α- and β-glucosidases which are involved in HIV infection. Unguilin was devoid of lectin and ribonuclease activities. It inhibited methyl-3H-thymidine uptake by mouse splenocytes and it weakly inhibited translation in a rabbit reticulocyte lysate system. Unguilin resembles mungin in some aspects, but differs from it in others.

KEY WORDS: Cyclophilin-like protein; antifungal; antiviral; peas.

1. INTRODUCTION

A variety of proteins have been purified from leguminous plants, including lectins with antitumor, immunomodulatory, and other activities (Kamemura et al., 1993), trypsin inhibitors with antitumor activity (Birk, 1985), antifungal proteins with different structures including thaumatin-like proteins (Ye et al., 1999), miraculin-like protein (Ye et al., 2000b), chitinases (Benhamou et al., 1993; Vogelsang and Barz, 1993; Ye et al., 2000a), and ribosome-inactivating proteins (Leah et al., 1991), in addition to a host of other proteins. Leguminous plants have drawn the attention of many investigators. Since different leguminous plants may elaborate different kinds of antifungal proteins (Ye et al., 1999, 2000a, b), the present investigation was undertaken to ascertain if the black-eyed pea produces a new antifungal protein. In the present report the finding of an antifungal protein carrying an N-terminal sequence displaying remarkable resemblance to that of cyclophilins, which have been described as a superfamily of ubiquitous folding catalysts (Goethel and Marahiel, 1999), is presented. The antifungal protein is an inhibitor of HIV-1 reverse transcriptase.

A cyclophilin-like antifungal protein was first reported from the mung bean (Ye and Ng, 2000). The present study provides evidence for the presence of this new class of antifungal proteins in another leguminous species, the black-eyed pea, and thus corroborates the defensive role played by the cyclophilin-like proteins.

2. MATERIALS AND METHODS

2.1. Isolation of Antifungal Protein

Black-eyed pea (Vigna unguiculata) seeds purchased locally were soaked in distilled water and homogenized, and the supernatant that was collected after centrifugation...
of the homogenate was dialyzed against distilled water. Tris–HCl buffer (pH 7.2) was added until its final concentration reached 10 mM. The crude extract was applied to a column of Affi-gel blue gel (Bio-Rad) (2.5 × 10 cm) which had been equilibrated and eluted with 10 mM Tris–HCl buffer (pH 7.2). After elution of the unadsorbed proteins, adsorbed proteins were eluted by NaCl gradient (0–500 mM) in the Tris–HCl buffer. Following dialysis against 10 mM Tris–HCl buffer (pH 7.2), the adsorbed proteins were loaded on a column of CM-Sepharose (1.5 × 18 cm). The column was eluted with the same buffer to remove unadsorbed materials. Adsorbed proteins were desorbed by NaCl gradient (0–500 mM) in 10 mM Tris–HCl buffer (pH 7.2).

2.2. Determination of Molecular Weight

For assessing the molecular weight of unguilin, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in accordance with the procedure of Laemmli and Farve (1973). The N-terminal sequences of unguilin were elucidated by employing a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC System.

2.3. Assay of Antifungal Activity

In the assay for antifungal activity, sterile petri plates (100 × 15 mm) containing 10 ml of potato dextrose agar were used. After the fungal colony had developed, paper disks (0.625 cm in diameter) were placed 0.5 cm from the rim of the colony. A 6-μl aliquot of the test sample in 10 mM Tris–HCl buffer (pH 7.2) was applied to each disk. Incubation of the petri plate was carried out at 23°C for 72 h until mycelial growth had enveloped peripheral disks containing the control and had generated crescents of inhibition around disks with antifungal samples. Three fungal species, Coprinus comatus, Mycosphaerella arachidicola, and Botrytis cinerea, were examined in the assay (Ye et al., 1999).

2.4. Assay of HIV-1 Reverse Transcriptase Inhibitory Activity

HIV-1 reverse transcriptase inhibitory activity was determined by ELISA following the method of Collins et al. (1997a), using a nonradioactive kit from Boehringer Mannheim (Germany). The inhibition assay was carried out as detailed in the procedure included with the kit, except that each well contained 2 ng of recombinant HIV-1 reverse transcriptase in a total reaction volume of 60 μl.

The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(A) · oligo(dT)15. In place of radiolabeled nucleotides, digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into the same DNA molecule, which is freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol: Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405 nm can be determined using a microtiter plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of unguilin was calculated as percentage inhibition compared to a control without the protein.

2.5. Assay of Glycohydrolase-Inhibitory Activity

The glycohydrolases α- and β-glucosidase are found in the Golgi complex and are associated with the processing of viral proteins. α-Glucosidase in particular is related to HIV-1 gp 120 glycosylation. Thus glycohydrolase-inhibitory activity is related to anti-HIV activity. In the assay for glycohydrolase-inhibitory activity, α-glucosidase, β-glucosidase, and β-glucuronidase and their corresponding β-nitrophenyl glucoside substrates were used to set up the enzymatic reaction in 10 mM MES buffer (pH 6.4) in a 96-well microplate. Solutions of substrates and enzymes were made in 50 mM buffer appropriate for each enzyme; MES-NaOH (pH 6.5) for α-glucuronidase, sodium acetate (pH 5.5) for β-glucosidase, and sodium acetate (pH 5.6) for β-glucuronidase. Each well of the microplate contained 2 mM substrate, 40 mM buffer, and enough enzyme to produce a measurable change in absorbance at 405 nm (0.2 unit/well for α-glucosidase, 0.01 unit/well for β-glucosidase, and 100 units/well for β-glucuronidase). Unguilin was allowed to interact with the enzyme for 5 min before the enzymatic reaction was initiated by addition of substrate. The total reaction volume was 0.2 ml. After incubation at room temperature for 15 min the reaction was brought...