Characterization and biological activities of Chenopodium leaf hemagglutinin (CLH)

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Received 27 March 2000; revised 31 July 2000

A hemagglutinin (CLH) having native molecular mass of 58 kDa and subunit molecular mass of 33 kDa had been purified from the leaves of Chenopodium amaranticolor. The protein agglutinated rabbit erythrocytes and no agglutination was observed with any of the groups A, B or O of human blood. The amino acid composition revealed that CLH was rich in aspartic acid, glutamic acid, glycine and phenylalanine and also significant amount of methionine. The N-terminal amino acid sequence analysis showed that CLH had no homology with any of the plant hemagglutinins studied so far. It was inactive towards human peripheral blood cells but mitogenic for mouse spleen B-lymphocytes. CLH inhibited protein synthesis in rat thymocytes at high concentration. CLH did not inhibit TMV infection of leaves indicating absence of antiviral properties.

Chenopodium (C. amaranticolor) is a universal local lesion host plant for many viruses. The lesion acts as a check for viruses spread by synthesizing pathogenesis-related (PR) protein and other defense proteins1. Earlier experiments on the induction of PR proteins in Chenopodium leaves in response to tobacco mosaic virus (TMV) revealed that they are presenting the intercellular space in the leaf tissue2. Interestingly, among the several PR proteins, a hemagglutinin [Chenopodium leaf agglutinin (CLH)] was also detected which was present in both normal as well as TMV infected leaves. On purification, the CLH was found to be a thermostable protein with activity at a broad range of pH 3-12 (ref. 3). The role of this particular hemagglutinin in the plant is not yet understood. In this paper, we report the sequence and composition of this homodimeric protein besides its various biological activities.

Materials and Methods

Leaves collected from 45-60 day old Chenopodium plants were used as biological material. All the chemicals and reagents used were of analytical grade. Fine chemicals and molecular weight markers for gel filtration were procured from Sigma Chemical Company, St Louis, Mo, USA. Liquid scintillation fluid (Cocktail W) was obtained from SISCO Research Laboratories, Mumbai, India, [14C]-Leucine and [3H]-thymidine were produced from BRIT, Mumbai, India.

Purification of Chenopodium leaf hemagglutinin

The methodologies used to purify Chenopodium leaf hemagglutinin (CLH) have been mentioned in the earlier paper3.

Amino acid composition

Amino acid analysis was performed by the method of Ashman and Bosserhoff4 in a Pharmacia HPLC instrument using dual pump binary gradient system and C-18 column. The protein sample was hydrolyzed in 1 ml of 6 N HCl at 110°C for 24 hr and the dried acid-free hydrolysate was dissolved in 100 μl of 0.25 M K-borate buffer, pH 10.4 before derivatization. The number of tryptophan residues were determined by the spectrophotometric method of Spande and Wittkop5.

Amino acid sequence analysis

The amino acid sequence analysis was carried out in the Indian Institute of Technology, Mumbai, India. The purified CLH showed single band of molecular weigh 33 kDa on SDS-PAGE. N-terminal sequence based on the Edman degradation reaction was performed in a Shimadzu PPSQ gas phase protein sequencer, using a C-18 column for reverse phase HPLC of phenyl thiodydantoin (PTH) amino acids,
and on-line PTH analyzer and a CR-7A data processor. Edman degradation reaction was performed on the purified CLH electrobotted onto a polyvinylidene difluoride (PVDF) membrane.

Chemical modification studies

Modification of histidine

Chemical modification of purified hemagglutinin was investigated in order to identify the amino acids involved at the active sites.

The modification of histidine was done by following the method of Melchior and Farney, the protein (100 μg) was taken in eppendorf tubes in a final volume of 200 μl with 20 mM Na-Phosphate buffer, pH 7.2. Diethyl pyrocarboxylic acid (15 μl) was added to the solution. The modified sample was then dialysed against 20 mM Tris-HCl, pH 8.0, at 4°C and the agglutination assay performed.

Modification of lysine

To 70 μl (200 μg) CLH, 130 μl 0.1 M Tris-HCl buffer, pH 8.0 was added. To this 15 μl of citraconic anhydride was added with constant stirring and the mixture was allowed to react for 30 min at room temperature. After the reaction the sample was dialysed against 20 mM Tris-HCl buffer, pH 8.0 and the agglutination activity was estimated.

Modification of tryptophan

Oxidation of CLH with NBS (N-bromo succinimide) was carried out according to the method of Spande and Witkop. The CLH sample of 70 μl (200 μg) was taken in a 1 ml cuvette and to it, 930 μl of 0.1 M Na-acetate buffer, pH 5.0 was added. An aliquot of 5 μl of 10 mM NBS in water was then added with rapid mixing. After 10 min, the absorbance at 280 nm was consecutive additions. The number of residues oxidized was calculated.

Modification of carboxyl groups

The hemagglutinin solution of 70 μl (200 μg) was dissolved in 1 ml of 1 M α-amino butyric acid methyl ester, pH 4.7. To it, 50 μl of 1.1 M 1-ethyl-3-(3-dimethyl aminopropyl)-carboxodimide hydrochloride (EDC) was added twice at an interval of 30 min. The mixture was stirred constantly and the pH at 4.7 by adding HCl. The reaction was carried out for 5 hr. the agglutination activity was estimated after dialyzing the sample against 20 mM Tris-HCl, pH 8.0.

Biological activities of CLH

Antiviral activity

The effect of CLH on the multiplication of TMV was assessed in Nicotiana glutinosa and Nicotiana tabacum (Xanthi NN). To assess the antiviral activity, TMV was mixed with CLH and was inoculated in such a manner that one-half of the leaf served as control (TMV only) and the other half was treated (TMV + CLH). Concentration of TMV was 21.6 μg/ml. The concentrations of CLH were 2.3 and 11.6 μg/ml in 10 mM phosphate buffer (PB), pH 7.0. The molecular ratio of CLH to TMV were 125 and 671 in the two experiments. In each set, three plants were selected and eight leaves from each plant were used for half-leaf inoculation as per the protocol mentioned above. N. glutinosa was left at room temperature, whereas Xanthi NN was left at 22°C. The number of lesions were counted on each half-leaf, two days after inoculation.

Mitogenic property

The mitogenic property of hemagglutinin was assessed by standard lymphocyte proliferation assay using spleen cells of C3H mice. Spleens were dissected from 10-12 week old female C3H mice and single cell suspension was prepared in RPMI 1640 tissue culture medium (Cat. No. R4130, Sigma Chemicals Co., USA) containing 15 mM HEPES buffer, 2 mM L-glutamine and 10% C-PDR 2 (controlled processed serum replacement, Cat. No. 9030, Sigma Chemical Co., USA). Erythrocytes were lysed by treating with 0.83 M NH₄Cl. Cells were incubated with different concentrations of hemagglutinin ranging from 0.43 to 37.5 μg/ml in a final volume of 200 μl containing 2×10⁵ cells in a 96 well Nuine microculture plate at 37°C for 72 hr in 5% CO₂ atmosphere. At the end of incubation, cells in each well were pulsed with I μCi of [³H]-thymidine (Specific Activity 6500 μCi/mmoll) for 16 hr. The cells were harvested on glass fibre filter discs and [³H]-thymidine incorporation was estimated using a liquid scintillation counter.

For assessing the mitogenicity of hemagglutinin to T and B lymphocytes, splenic lymphocytes were separated into T-cell rich (non-adherent) and B-cell rich (adherent) sub-populations by fractionation on ‘nylon wool’ columns essentially according to the method of Julius et al. The column was prepared by packing 300 mg wool (Leucopak, Fannwall Labs, USA) a 5 ml sterile plastic syringe and was equilibrated with RPMI 1640 medium containing
10% foetal calf serum. Fifty million splenic lymphocytes were loaded on the column and it was incubated in 5% CO₂ incubator at 37% for 30 min. The elution was done with 2 volumes of prewarmed medium. After the collection of non-adherent fraction (T-cell rich), the column was washed with 10 volumes of the medium and adherent cells (B-cell rich) were obtained by repeated flushing of the column with fresh medium. The separated T and B-cell rich fractions were incubated with hemagglutinin for mitogenic assay as described above. Con A and LPS (E.coli) were used as positive controls for T-cell and B-cell mitogenesis, respectively.

**Effect of CLH on in vivo protein synthesis in thymocytes**

Thymocytes were isolated from 5 weeks old rat. The cells were suspended in RPMI 1640 medium. The density was estimated to be 155x10⁶ cells/ml. A pulse of 0.5 μCi of [¹⁴C]-leucine in a final volume of 1.5 ml was given (1 ml cells, 5 μl [¹⁴C]-leucine of 0.5 μCi and 495 μl PBS). The cells were incubated from 30-180 min at an interval of 30 min. Two independent treatments were given using 40 µg and 80 µg of CLH. The reaction was stopped by adding TCA to a final concentration of 10%. The precipitated protein was adsorbed on Whatman 3 filter paper discs by suction. The disc was washed thrice with 10% TCA followed by acetone. The discs were then air dried and used for radioactive counting.

**Results**

**Properties of purified Chenopodium leaf hemagglutinin**

**Amino acid composition of CLH**

Amino acid composition of CLH is listed in Table 1. The CLH contained a large amount of acidic amino acids like aspartic acid, glutamic acid and glycine which amount to be 48% of the total amino acid content. The hemagglutinin also contained a higher amount of methionine as compared to other seed or leaf agglutinins. The phenylalanine content was found to be considerably high.

**Amino acid sequence analysis**

The sequence of 15 amino acids starting from N-terminal of the purified CLH subunit (33 kDa) was determined. The deduced sequence from the data was found to be Asn-Glu-Glu-Lys-Val-Tyr-Ile-Thr-Ile-Gly-Gly-Leu-Gly-Asp-Asp.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mole percentage</th>
</tr>
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<tbody>
<tr>
<td>Aspartic acid</td>
<td>22.4</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>12.1</td>
</tr>
<tr>
<td>Serine</td>
<td>4.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>13.4</td>
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<td>Threonine</td>
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</tr>
<tr>
<td>Tyrosine</td>
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</tr>
<tr>
<td>Methionine</td>
<td>4.3</td>
</tr>
<tr>
<td>Valine</td>
<td>5.8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>11.5</td>
</tr>
<tr>
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</tr>
<tr>
<td>Leucine</td>
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</tr>
<tr>
<td>Lysine</td>
<td>1.5</td>
</tr>
<tr>
<td>Cysteine</td>
<td>3.2</td>
</tr>
<tr>
<td>Tryptophan*</td>
<td>3.7</td>
</tr>
</tbody>
</table>

*Number of tryptophan residues were calculated spectrophotometrically.

**Chemical modification studies of CLH**

More than 90% of agglutination activity was lost when lysine residue was modified with citraconic anhydride. Similar results were obtained when histidine was modified with diethyl pyrocatecholate. From the sequential oxidation of tryptophan which involved about 12 residues using NBS, about 90% loss of agglutination activity was observed (data not shown). The results reveal that lysine, histidine and tryptophan are involved at the active sites. However, CLH lost only 55% of its activity when carboxyl groups were modified with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide followed by coupling with ε-amino butyric acid methyl ester.

**Mitogenicity of CLH**

The hemagglutinin was mitogenic to mouse spleen cells (Fig. 1a; Stimulation Index (SI) = 1.9 at 37 µg/ml). Enrichment of spleen cells in B lymphocytes (Nylon adherent subpopulation) significant improved their response to the hemagglutinin (SI = 8.2 at 37.5 µg/ml). On the other hand, enrichment in T-cells did not improve their response in comparison to that of unfraccionated spleen cells. These data suggest that CLH was likely to be a polyclonal B-cell mitogen, though less potent than LPS (Fig. 1a). It did not show any mitogenic activity towards human peripheral blood lymphocytes (Fig. 1b).
Effect of CLH on in vivo protein synthesis in thymocytes

In order to understand the effect of CLH on protein synthesis, the study of its action on rat thymocytes was undertaken. At two different concentrations of CLH used, there was no marked inhibition of in vivo protein synthesis in the presence of CLH. However, at higher concentration of CLH (80 μg), 25% inhibition was observed (Fig. 2).

Anti-viral activity of CLH

Three plants were selected and eight leaves from each plant were used for half-leaf inoculation for each set of experiment. Number of lesions was counted after 48 hr of inoculation and the data are shown in Table 2. The result showed that CLH did not inhibit TMV infection which reveals that it was not an antiviral agent.

Discussion

CLH is a powerful hemagglutinin with a specific activity of about 160 agglutination units per μg of protein. This hemagglutinin is synthesized exclusively in the leaves. Chenopodium seeds do not contain a hemagglutinin which either agglutinate rabbit erythrocytes or cross-react with CLH-antibodies. The differences between hemagglutinin in seeds and in other vegetative parts have been reported.

Fig. 1—Mitogenic activity assay of CLH by the method of [3H]-thymidine incorporation. [(a): [3H]-thymidine incorporation using mice spleen cells. (c), Control (only cells); (o), total spleen cells; (●), enriched B-cells; (△), enriched T-cells. (b): [3H]-thymidine incorporation using human lymphocytes. (c), Control (only cells); (o), human peripheral lymphocytes.]
agglutination activity was found with trypsin and protease K resulted in a significant destruction of CLH. However, no loss of agglutination activity was found with trypsin treatment (data not shown). Also, no protease inhibitor activity was observed when CLH was incubated with either trypsin or Helicoverpa armigera gut extract (data not shown).

The treatment with proteases like pronase, papain, and proteinase K resulted in a significant destruction of agglutination activity of CLH. However, no loss of agglutination activity was found with trypsin treatment (data not shown). Also, no protease inhibitor activity was observed when CLH was incubated with either trypsin or Helicoverpa armigera gut extract (data not shown).

optimal concentration of CLH (Fig.1). Since isolated T-cells did not show any stimulation, CLH can be considered as a B-cell mitogen. No mitogenic activity was observed towards human peripheral blood lymphocytes. No mitogenic leaf hemagglutinin has been reported so far, for which the hemagglutinin has got a complex carbohydrate specificity. An earlier report showed that pokeweed root lectin, Pa-1 was mitogenic for human and murine T and B-cells.

The degree of mitogenicity of CLH was found to be comparable with that of Pa-1. CLH is a dimer and it has been suggested Waxdal and Basham that B-cell activation requires the interaction of several cell receptors with multimeric stimulants. CLH is the first hemagglutinin which has a complex carbohydrate specificity and at the same time, it is mitogenic specifically for mouse spleen B-cells.

The studies on in vitro protein synthesis using rat thymocytes suggested that CLH is not a highly potent inhibitor of protein synthesis. However, at higher concentration of CLH (80 μg), 25% inhibition protein synthesis was observed. The CLH agglutinated thymocytes isolated from rat. Strong agglutination was observed with ascites cells from mice also (data not shown).

Several plant hemagglutinins are potent inhibitors of viruses which have glycoproteins in their virions. CLH, though found among the proteins present in the intercellular space of TMV infected leaf tissues, is not an anti-viral protein for TMV. Chenopodium is reported to contain an anti-viral protein in its leaf. Like many other plant species, Chenopodium amaranticolor also contains a single chain RIN (ribosome inactivating protein) The amino acid sequence analysis CLH did not reveal significant homology with any of the leaf or seed phytohemagglutinins so far described. The B-cells specific CLH showed a unique amino acid composition and amino acid sequence.

Acknowledgement

The authors wish to thank Dr. A. S. Bhagwat, Molecular Biology and Agriculture Division, Bhabha Atomic Research Centre, Mumbai, India, for his help in amino acid analysis using HPLC instrument.

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