Enzyme-Linked Immunosorbent Assay for Humic Acids

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An enzyme-linked immunoassay (ELISA) for the determination of humic acid in natural-water samples was developed. For antisera production, humic acid was coupled to helix pomatia hemocyanin. A humic acid-horseradish peroxidase conjugate was synthesized as a tracer. The detection limit of this competitive assay was estimated to be 0.5 µg/l, which means 100 pg absolute. The cross-reactivities at the center point (50%-value) of several commercial humic acids and both aliphatic and aromatic acids were determined. Moreover, the structural aspects of the humic acids could be elucidated.

Keywords Humic acid, enzyme-linked immunoassay, polyclonal antibody, cross-reactivity, dissolved organic carbon, water analysis

Enzyme immunoassays have been found to be useful for a rapid, convenient and accurate detection of environmental pollutants¹⁻⁷ (e.g. pesticides, polycyclic aromatic hydrocarbons and nitro aromatics).

Up to now, no immunoassay for macromolecules of natural origin, such as humic acids (HA)⁸, has existed. Humic acids are ubiquitous in natural water, and often exceed 50% of the dissolved organic carbon (DOC).⁹ The determination as well as quantification of humic acids is especially important for the treatment of drinking water, because the possible toxicological effects of chlorinated HAs are not completely understood at present. Furthermore, many compounds, as well as heavy metals¹⁰, form complexes with humic acids, and seem to be involved in the transport processes within the aquifer. At present, no analytical method exists to discriminate between native DOC and DOC that derives from anthropogenic contamination. In combination with chlorination and activated charcoal, humic substances are possible precursors for chlorinated hydrocarbons.¹¹

Due to their indefinite molecular weight, the heterogeneity of their structure and composition¹², the characterization of humic acids using conventional instrumentation is rather difficult.

In this communication an immunological method for the determination of HA is presented. With this competitive immunoassay a sensitive quantification of humic acids is possible. ELISA offers a faster, more economic technique compared to conventional instrumentation, such as fluorescence spectroscopy¹³⁻¹⁵, HPLC with

fluorescence detection¹⁶, photometrical methods¹⁷ and elemental analysis.¹⁸

Experimental

Chemicals

Humic acid was prepared by extracting a soil sample taken from a local forest (Buchendorf, Southern Germany) with potassium hydroxide according to a standard procedure.¹⁹ Helix pomatia hemocyanin for the immunization was purchased from Serva (Heidelberg, Germany) and horseradish peroxidase was available from Boehringer (Mannheim, Germany). The other chemicals were from Fluka (Neu-Ulm, Germany), while the pesticide standards were purchased from Ehrenstorfer (Augsburg, Germany). The standard solutions were obtained by dilution with distilled and UV-treated water. An adjuvant for the immunization was taken from Gerbu (Heidelberg/Gaiberg, Germany).

Preparation of the immunogen

HA was conjugated to helix pomatia hemocyanin (HPH) via NHS-ester.²⁰ After 100 mg of HA was dissolved in 4 ml of dry dimethyl sulfoxide, 100 mg of *N*-hydroxysuccinimide (NHS), 700 mg of di(*N*-succinimidyl)carbonate (DSC) and 400 mg of *N*-(dimethylaminopropyl)-*N'*-ethylcarbodiimidehydrochloride (EDC) were added. The mixture was stirred at room temperature overnight. Then, after 100 mg of HPH was dissolved in 2 ml of a sodium borate buffer (0.1 M, pH 9 to 9.5), 200 µl of HA-NHS-ester was added. After 75 min the precipitate was separated via centrifugation. Two 500 µl-portions of activated HA were added to the supernatant. All precipitates were pooled, lyophilized

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and sieved (ϕ 63 μ m).

Antisera production

For immunization with the HA-CO-NH-HPH conjugate, an adult random-bred rabbit was injected with a mixture of immunogen and (*N*-acetylglucosaminyl-(β 1-4)-*N*-acetylmuramyl-L-alanyl-D-isoglutamin, GMPD) according to the instructions of the manufacturer. One milligram of immunogen was then suspended in 0.5 ml of physiological saline. To this mixture 20 μ g of adjuvant was added and thoroughly shaken until a homogeneous suspension was formed. The immunogen was injected subcutaneously at one position of the animal's back. In the same manner, booster injections were administered 8, 16, 22 and 31 weeks later. The rabbit was bled through the ear vein within 7 to 14 d after each booster injection. Either serum or plasma was obtained and divided in 1 ml aliquots and then stored undiluted after the addition of 0.02% NaN₃ at -20°C until use.

AK 392: HA-CO-NH-HPH as immunogen.

Preparation of the humic acid-enzyme conjugate²¹

After 4 mg of horseradish peroxidase (POD) was dissolved in 330 μ l of UV-treated water and NaIO₄ was added (200 μ l, 0.1 M), the mixture was stirred for 20 min at room temperature and 33 μ l of ethylene glycol was added in order to interrupt the oxidation procedure. Glutaric acid dihydrazide (100 μ l, 0.1 M) was then added. Two hours later, a gel chromatography was performed on a Sephadex PD-10 column, equilibrated with phosphate buffered saline (PBS) in order to separate free glutaric acid dihydrazide from the POD-conjugate. An empty microtiter plate was taken as a manual fraction collector. The cavities with the highest absorptions at 450 nm were pooled.

Then, 100 μ l of a HA solution (10 g/l in sodium borate buffer, 0.1 M, pH 9 to 9.5) was added to about 800 μ l of activated POD. After stirring for 12 h at room temperature the tracer was purified on a Sephadex PD-10 column, equilibrated with PBS (pH 7.6).

To stabilize the conjugate, a crumb of thymol was added.

ELISA procedure

The assay procedure and calculation were performed as previously described.^{3,6} Microtiter plates were coated with sera diluted 1:30000 in a coating buffer (pH 9.6). A calibration curve of HA was prepared ranging from 0.001 – 1000 μ g/l in a phosphate buffer. After incubation of the analyte (200 μ l/cavity) an additional washing step (PBS/Tween) was performed using an eight-channel washer. The enzyme tracer was then added, diluted (1:100000) in PBS (pH 7.6). After incubating for 15 min and undergoing an additional washing step, a substrate solution was added. The enzymatic reaction was stopped by adding sulfuric acid. Finally, the absorbance at 450 nm was measured using a microtiter plate reader.

Specificity of the antibody

The specificity of the antibodies was described while determining the cross-reactivity (CR),

$$CR[\%] = \frac{50\%-value \text{ ("standard"-HA)}}{50\%-value \text{ (related compound)}} \times 100,$$

of different humic acids and an arbitrary selection of both aliphatic and aromatic acids. Some of these substances are supposed to appear as structural elements^{12,22,23} in humic acids.

Results and Discussion

Standard calibration curve

Figure 1 shows a standard curve for humic acid with the polyclonal antibody (AK 392). The absorbance at 450 nm is plotted versus the concentration on the logarithmic scale. The calibration solutions of the humic acid ($n=3$) range from 1 ng/l to 1 mg/l; the error bars correspond to the range (2s). The detection limit (d.l. = $\bar{x}_B + 3s_B$) for the "standard" humic acid was estimated to be about 0.5 μ g/l, which means 100 pg absolute. The center point (50%-value) was determined to be 13 μ g/l.

Specificity

The cross-reactivities of some humic acids and both aliphatic and aromatic acids are given in Table 1. Antibody AK 392 can recognize several humic acids of different origin. Since the cross-reactivities are between 77 to 127%, water samples can be examined regarding their humic-acid concentration.

On the other hand, the aliphatic and aromatic acids as well as EDTA and the acidic polymer can provide some information concerning the structure of the humic acid

Fig. 1 Calibration curve for humic acid "Buchendorf" ($n=3$, 2s); the center point is 13 μ g/l, and the detection limit 0.5 μ g/l.

Table 1 Cross-reactivities with humic acids and other compounds

Analyte	Cross-reactivity, %
Humic acid ^a (Buchendorf) "standard"	100
Humic acid (Aldrich)	77
Humic acid (Fluka)	109
Humic acid (Roth)	127
Humic acid (Bad Elster)	84
Ascorbic acid ^b	<0.1
Barbituric acid ^b	<0.1
Citric acid ^b	<0.1
EDTA·2Na	<0.1
Ferulic acid ^b	<0.1
Polymaleic acid ^b	<0.1
Sodium salicylate ^b	<0.1

a. All HAs were treated with 0.1 M KOH, precipitated with 5% HCl and washed several times with distilled water before using. Stock solutions were prepared in a coating buffer (pH 9.6, 1 g/l), while dilutions (1 ng/l–1 mg/l) were made in PBS.
b. Standard solutions as well as dilution curves were prepared in PBS.

used as the immunogen. The results demonstrate that these molecules either do not have high structural similarities to the humic-acid structure, or that they could not bind to all of the important antibody types at one time. The result with the anionic polymer shows that the assay is not based on pure electrostatic interactions, but requires the important features of humic acids to produce a positive response.

The results illustrate in a clear way the presence of antibodies that detect humic acids, so a new method has been opened for the detection of humic acids. The sensitivity of the immunoassay is about 0.5 µl/l; thus, natural-water samples can be examined without any clean-up step and enrichment. The humic-acid concentrations found in water¹⁶ vary between 0.6 mg/l (inshore seawater) and 8.0 mg/l (river water). Higher concentrations (<30 mg/l) have been found in bog water.

Furthermore, this assay shows that antibodies would be valuable tools for elucidating the local and general structure of humic substances, as well as related compounds regarding their heterogeneity and complexity. This might be the way out of the fundamental stagnation which has been noticed in the field of humic-acid research for many years.

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