Eclética Química

Print version ISSN 0100-4670*On-line version* ISSN 1678-4618

Eclet. Quím. vol.27 no.special São Paulo 2002

http://dx.doi.org/10.1590/S0100-46702002000200003

Preparation and evaluation of atrazine immunoconjugate

Preparo e avaliação do imunoconjugado para atrazina

Carla dos Santos Riccardi[I]; Ângela Regina Araújo[II];Hideko Yamanaka[I]

[I]Departamento de Química Analítica - Instituto de Química - UNESP - 148-01-970 - Araraquara - SP

[II]Departamento de Química Orgânica - Instituto de Química - UNESP - 148-01-970 - Araraquara - SP

ABSTRACT

In order to study the affinity reaction between the anti-atrazine antibody and atrazine, an enzyme was incorporated, as a marker, to an atrazine carboxylic derivative. The hapten and conjugate were synthesized and characterized by MS, IR and NMR. The interaction between monoclonal antibodies and hapten-HRP conjugate was investigated by enzyme linked immunosorbent assay (ELISA).

Keywords: atrazine, conjugate, ELISA, environmental monitoring

---

RESUMO

A fim de estudar a reação de afinidade entre os anticorpos anti-atrazina e a atrazina, uma enzima foi incorporada, como marcador, a um derivado carboxílico da atrazina. O hapteno foi caracterizado por espectometria de massa (EM), infravermelho (IV) e ressonância magnética nuclear (RMN). A interação entre anticorpos monoclonal e o conjugado de hapteno-HRP foram determinados pelo ensaio imunoenzimático (ELISA).

Palavras-chave: atrazina, conjugado, ELISA, monitoramento ambiental

Introduction

The initial delineation stages of an immunoassay involve the selection of the target molecule; preparation of the hapten, which consists in the synthesis of the target molecule derivative containing a group for attachment to the protein; covalent binding of the hapten with enzyme to form a hapten-enzyme conjugate; and finally the affinity reaction.

The ideal hapten for a selected target analyte molecule has to be a nearperfect mimic of that molecule, both in structure and geometry, in electronic and hydrogen-bonding capabilities, and in hydrophobic properties. The hapten should contain a "handle", terminated with a functional group capable of covalent bonding to enzyme. Common functional groups are –COOH, -OH, $-NH_2$ or –SH[3]. The small hapten molecules require to the spacer group between enzyme and hapten molecule in order to improve the recognition sites with antibody.

Materials and methods

Chemicals

Isopropylamine, *N, N*-diisopropylethylamine, cyanuric chloride (2,4,6-trichloro-1,3,5-triazin), *N*-hydroxysuccinimide (NHS), *N, N*□-dicyclohexylcarbodiimide (DCC), Tween 20, bovine serum albumin (BSA) and peroxidase (HRP) (EC 1.11.1.7) type VI-A from horseradish, 1100 U/mg were purchased from Sigma; 6-aminohexanoic acid, anhydrous dimethylformamide (DMF) and ethylenediaminetetracetic acid (EDTA) were purchased from Merck. Other reagents were analytical grade.

The monoclonal antibodies (mAb, clone K4E7) were given by Prof. Dr. B. Hock, Technical University of München, Freising.

Apparatus

Mass spectra (MS) was obtained on a VG-Platform-Fisons spectrometer (VG Analytical, Wythenshawe, U. K) using 70-eV ESI for ionization and data are reported as m/z (relative intensity). Infrared spectra (IR) was measured on a FTIR Perkim Elmer 2000 spectrometer. ^{1}H and ^{13}C: NMR spectra were obtained with a General Electric AC 200 F (Bruker NMR, Billerica, MA) opering at 200 MHz for ^{1}H and 75 MHz for ^{13}C. Chemical shifts (d) were expressed in parts per million using tetramethylsilane as an internal standard. Thin Layer Chromatography (TLC) was performed on 0.25 mm, pre-coated silica gel 60 F254 aluminum (Merck, Gillstown, NJ). Compounds were detected by exposure to iodine vapor; eluent systems were described in the individual experiments. ELISA (Enzyme Linked ImmunoSorbent Assay) was performed in 96-well microplates (COSTAR) with Organon Teknika 2001 reader.

Synthesis of Hapten

Hapten (Hp) was synthesized by the routes as shown in Figure 1, following the procedure described by Goodrow et al.[2]. The resulting hapten was characterized by MS, IR, and ^{1}H and ^{13}C:NMR.

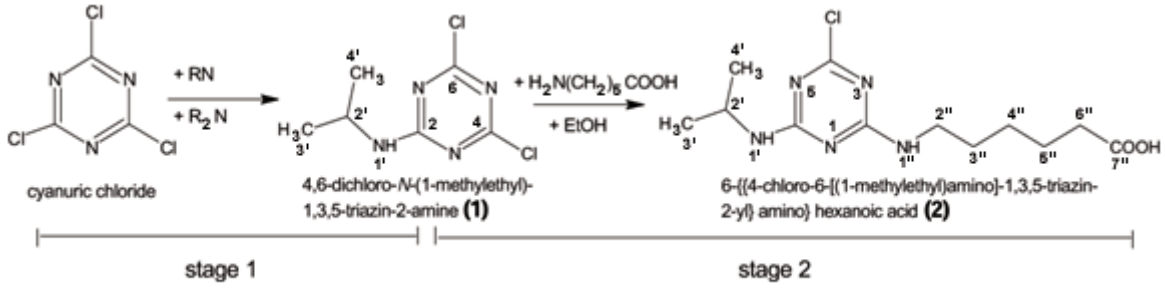

FIGURE 1 – Synthesis and structure of atrazine carboxylic derivative for conjugation to a linked enzyme.

To 50.0 mmol of cyanuric chloride in 400 mL of ether cooled to – 20 ^{0}C was added, over 45 min, 52.0 mmol of isopropylamine (70 wt% in water) and 52.0 mmol of *N, N*□-diisopropylamine in 50 mL of ether. The mixture was filtered, and the filtrate was washed sequentially with 1 mol L^{-1} HCl (25 mL), 5 % m/v $NaHCO_3$ (25 mL), and satured NaCl (2 x 25 mL) and then dried (Na_2SO_4). The ether was removed under reduced pressure, leaving a pale yellow oil (Figure 1, compound **1**). This compound reacted with 6-aminohexanoic acid (20.0 mmol) and the compound **2** (Figure 1) was obtained. The purity of both compounds were investigated by TLC (Thin-Layer Chromatography, chloroform/methanol 1:1 v/v).

Preparation of the enzyme tracer

The hapten-HRP conjugate was synthesized using the method described by Pradelles[5]. 20 mL of *N*-hydroxysuccinimide (200 nmol) and 20 mL *N, N*□-dicyclohexylcarbodiimide (200 nmol), both dissolved in anhydrous dimethylformamide (DMF), were successively added to 20 mL of atrazine carboxylic derivative solution in DMF. After incubation for 4h at room temperature, 60 mL of this mixture was added to 600 mL of HRP (1 mg mL^{-1}) dissolved in 0.1 mol L^{-1} borate buffer solution (pH 8.5). The reaction was processed for 30 min at room temperature and then it was stopped by the addition of 400 mL of 0.1 mol L^{-1} phosphate buffer solution (pH 7.4) containing 0.4 mol L^{-1} NaCl, 1 mmol L^{-1} EDTA, 0.1 % m/v bovine serum albumin and 0.01 % m/v sodium azide. The obtained mixture was dialyzed for 12h against 0.01 mol L^{-1} borate buffer solution (pH 8.5) and 4h against 0.03 mol L^{-1} phosphate buffer solution (pH 7.5). Aliquot of the Hp-HRP were stored frozen.

Elisa (Enzyme Linked ImmunoSorbent Assay) development

The Hp-HRP conjugate was evaluated by ELISA. The 96-well microtiter plates were coated with 100 mL mAb specific for atrazine, prepared in 50 mmol L^{-1} carbonate buffer solution (pH 9.6), at 4 ^{0}C overnight. The plates were washed three times with phosphate buffered saline (PBS)/Tween 20 solution (50 mmol L^{-1} pH 7.6, 0.05 % m/v Tween 20). Then the plates were incubated for 1h with PBS/Tween 20 solution containing 0.1 % m/v BSA. After a second similar washing step, 100 mL

enzyme tracer were added and incubated for 1h at 37 ^{0}C. Sequentially the plates were washing and 100 mL substrate solution (3, 3', 5, 5'-tetramethylbenzidine and ureia peroxide) were added. The enzymatic reaction processed for 20 min. Then were added 100 mL reaction stop solution (1 mol L^{-1} sulfuric acid) to each well and the absorbance were measured at 450 nm. All steps were carried out at room temperature except for mAb coating and affinity reaction stages.

Results and discussion

Hapten Synthesis

The development of immunoassays and the preparation of haptens for atrazine or other *s*-triazines has been well documented [1,2, 4, 6]. Different types of spacer groups were used to synthesize the immunogen and tracer. This can help to avoid problems associated with antibodies directed to the spacer group rather than to the hapten itself. In some cases the use of heterological spacers improves the sensitivity of the assay. The haptens with C6 spacer arms conjugated to HRP resulted in 3-5 fold increased sensitivity compared to the haptens with C3 spacer arms. In general, HRP tracers with the long spacer arms resulted in the most favorable assay conditions. This hapten has a C6 spacer that mimics the ethyl group of the atrazine. At the same time, important antigenic determinants in the atrazine chemical structure such as the bulky isopropylamine group and the electronegative chlorine atom remains distant from the shielding effect caused by the protein [2].

The synthesized compounds were detected on TLC by staining in an iodine chamber. TLC Rf 0.51 e 0.13 to compounds **1** and **2** (white pasty, mp 160-161 ^{0}C), respectively. Structural confirmation was accomplished by MS, IR, ^{1}H and ^{13}C: NMR (Proton-Desacopled ^{13}C and Distortionless Enhancement by Polarization Transfer (DEPT) experiments). All of these date were consistent with the assigned structure for the compounds 1 and 2. From the mass spectra it was observed *m/z* 206 and 302, together with the peaks [M + 1] e [M + 2] regarding to the presence halide in the molecule (Cl isotopic). IR (KBr) bands had been found n N-H (3400 / 3450 cm^{-1}), d N-H (1620 / 1580 cm^{-1}), n C-H (< 3000 cm^{-1}) and n C-Cl (745-695 cm^{-1}) (compounds **1** e **2**, respectively); n C=O (1698 cm^{-1}) (compound **2**). The presence of the atrazine carboxylic derivate was confirmed by ^{1}H and ^{13}C:NMR (Table 1), and also by direct comparison with the respective literature data [2]. The ^{1}H: NMR interpretation was initiated by analysis of the alifatics chains of compound **1** (*N*-alkyl-amino group) and compound **2** (*N*-alkyl-aminohexanoic group). Finally, ^{13}C:NMR experiments confirmed the presence of the carbon atoms of the heterocycle and hexanoic acid chain.

TABLE 1 – ^{1}H-NMR Data of Compounds **1** ($CDCl_3$, 200MHz) and **2** (DMSO-d_6, 200MHz)[2].

Carbon	1			2		
	^{1}H		^{13}C	^{1}H		^{13}C
	δ_H	mult J	δ_C	δ_H	mult J	δ_C
2			166.00			165.40
4			168.50			169.04
6			164.83			164.50
1´	7.27	sl		7.60	brs	
2´	4.23	m	43.70	3.99	m	42.98
3´	1.24	d (6.61Hz)	21.80	1.12	d (6.40Hz)	21.65
4´	1.26	d (6.61Hz)	21.94	1.14	d (6.40Hz)	21.96
1´´				7.80	brs	
2´´				2.19	m	33.59
3´´				1.50	m	24.14
4´´				1.30	m	26.59
5´´				1.50	m	28.34
6´´				3.20	m	39.80
7´´						174.43
- OH				11.90		

Elisa

On these experiments it was observed the ability of mAc (K4E7) to a binding with hapten-HRP conjugate. The linear range of 25-200 mg mL^{-1} (Figure 2) was verified for the conjugate concentrations by interaction with the monoclonal antibodies (10 mg mL^{-1}). Above of this value, the enzymatic immunoreaction tends to the saturation of the bonds sites by monoclonal antibodies (exponencial response: 200-240 mg mL^{-1}).

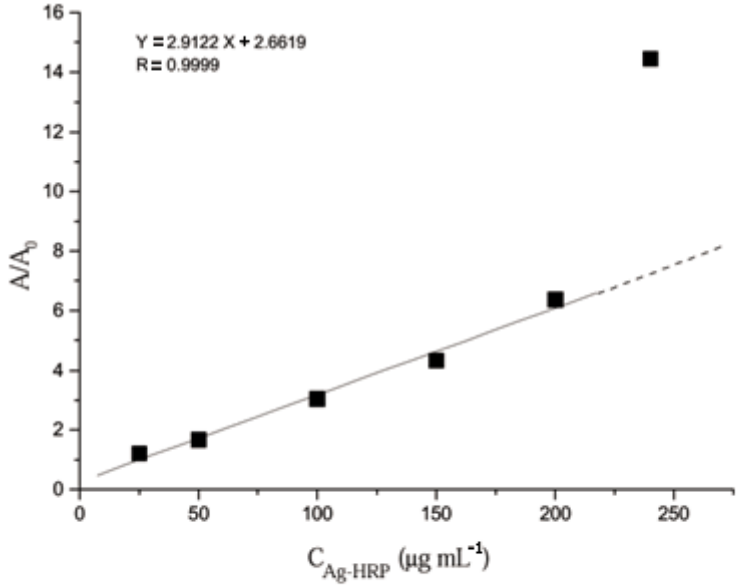

FIGURE 2 – Analytical curve of ELISA used to evaluate the binding of Hp-HRP conjugate. Microplates coated with fixed concentration of the antibodies 10 µg mL^{-1} were exposed to differents concentrations of conjugated.

Conclusions

The atrazine derivate was convenient synthetized and the hapten-HRP conjugate was suitable for the recognition with the anti-atrazine antibodies. Further, it will be used for the amperometric immunosensor investigation.

Acknowledgement

We thank Prof. Dr. B. Hock for giving the monoclonal antibodies; Prof. Dr. P. I. Costa, for helping on the immunoassays investigation; Department of Organic Chemistry by disposing the equipments for the compounds characterization and FAPESP for the fellowship.

References

1 DUNBAR, B.; RIGGLE, B.; NISWENDER, G. Development of enzyme-immunoassay for the detection of triazine herbicides. *J. Agric. Food Chem.*, v. 38, p.433-437, 1990. [Links]

2 GOODROW, M. H.; HARRISON, R. O.; HAMMOCK, B. D. Hapten synthesis, antibody development, and competitive-inhibition enzyme-immunoassay for s-triazine herbicides. *J. Agric. Food Chem.*, v.38, p.990-996, 1990. [Links]

3 HENNION, M. C.; BARCELO, D. Strengths and limitations of immunoassays for effective and efficient use for pesticide analysis in water samples: a review. *Anal. Chim. Acta*, v.362, p.3-34, 1998. [Links]

4 LUCAS, A. D.; BEKHEIT, H. K. M.; GOODROW, M. H.; JONES, A. D.; KULLMAN, S;. MATSUMURA, F.; WOODROW, J. E.; SEIBER, J. N.; HAMMOCK, B. D. Development of

antibodies against hydroxyatrazine and hydroxysimazine - application to environmental-samples. *J. Agric. Food Chem.*, v.41, p.1523-1529, 1993. [Links]

5 PRADELLES, P.; GRASSI, J.; CHABARDES, D.; GUISO, N. Enzyme immunoassays of adenosine cyclic 3', 5'-monophosphate and guanosine cyclic 3', 5'-monophosphate using acetylcholinesterase. *Anal. Chem.*, v.61, p.447-453, 1989. [Links]

6 WITTMANN, C.; HOCK, B. Development of an ELISA for the analysis of atrazine metabolites deethylatrazine and deisopropylatrazine. *J. Agric. Food Chem.*, v.39, p.1194-1200, 1991. [Links]

Recebido em 12.11.2001

Aceito em 10.12.2001