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EJ087199900065

Journal of Food and Drug Analysis
1999. 7(1) : 65-72

藥物食品分析
第七卷　第一期

The Synthesis of Antigens and the Production of Antibodies against Patulin Derivatives

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ABSTRACT

Patulin hemisuccinate (P-HS) was synthesized and conjugated to bovine serum albumin (BSA) as the hapten-protein conjugation for immunization. Polyclonal antibodies were obtained from the antiascites of six immunized mice. Titers and specificity were determined using an indirect ELISA. The results indicated that three of the six mice were better immunized and that the antibodies acted strongly against patulin-hemisuccinate-BSA. Slight ELISA responses of the antibodies against patulin alone showed the potential for developing monoclonal antibodies.

Key words: **patulin derivatives, polyclonal antibody.**

INTRODUCTION

Patulin is one of the most important mycotoxins in horticultural products. It is usually found in apple products (apple juices, pastes, or ciders) resulting from the contamination of *Penicillium* or *Aspergillus* before manufacturing[1]. Patulin has been proven to be a chemotherapeutic agent to microorganisms and has been demonstrated to have carcinogenicity to many animals[1,2]. In some literature, chromatography methods for patulin analysis including thin layer chromatography[3] (AOAC method 974.18), liquid chromatography, gas chromatography and bioassay have been described[2]. However, the lowest determinable levels of these methods without derivation are higher than the official limit (50 ppb) set by the World Health Organization (WHO) in 1985[4,5]. Our laboratory had established a new method which employs direct acylation in samples, then followed by GC-MS determination[4]. The extensive protocols of sample cleanup and patulin derivation as well as the expensive analytical equipment make it difficult to extend the application outside the laboratory.

Immunochemical analytical methods have been shown during the past few years to have potential for routine analysis of many mycotoxins [2, 6, 7, 8]. The advantages of high specificity and high sensitivity as well as time-savings and no need for sample cleanup enable immunoassay to compete with chromatograph analytical methods in many ways[2, 7, 8]. Unfortunately, there still is no immunoassay available for patulin analysis.

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Accepted for Publication: Nov. 24, 1998

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Perhaps there exists a theoretically conflictive problem for the production of antibodies against patulin. Generally, a compound such as patulin with a low molecular weight (MW 154) is a non-proteinaceous toxin, and it is necessary to use a hapten-protein conjugate for antibody preparation and as a specific marker[9]. But the sensitivity of patulin to thio-containing compounds and basic conditions[1] might result in decomposition of patulin when conjugating it to the protein carrier. The exposed epitops of the injected hapten-protein conjugates can also be attacked by thio-containing proteins in the blood. This maybe the reason why there is only one report[2] demonstrating the production of antibodies against patulin derivatives and why there is no commercial product in the market. Weiss and McElroy (1993) conjugated patulin hemiglutarate to bovine serum albumin (BSA) and immunized rabbit to produce polyclonal antibodies[2]. However, the structure of the hapten patulin hemiglutarate was not clearly verified, and the specific antibodies against patulin alone were not sufficiently described in their report.

In this study, patulin and hapten patulin hemisuccinate (P-HS) were synthesized and verified. In addition, three kinds of patulin-protein conjugates were prepared using mixed anhydride and water-soluble carbodiimide methods. The production of polyclonal antibodies and qualitative assays by an indirect ELISA were also performed.

MATERIALS AND METHODS

I. *Material and Instrument*

Patulin was synthesized using methods described by Seijas *et al.* (1989)[10] and Bennett *et al.* (1991)[11] with a slight modification. All the solvents, ethylenediamine (EDA) and 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDPC), were obtained from E. Merck (Schuchardt, Germany). Succinyl chloride, bovine serum albumin (BSA), ovalbumin (OVA), phosphate buffered saline (PBS, 10 mM phosphate buffer pH

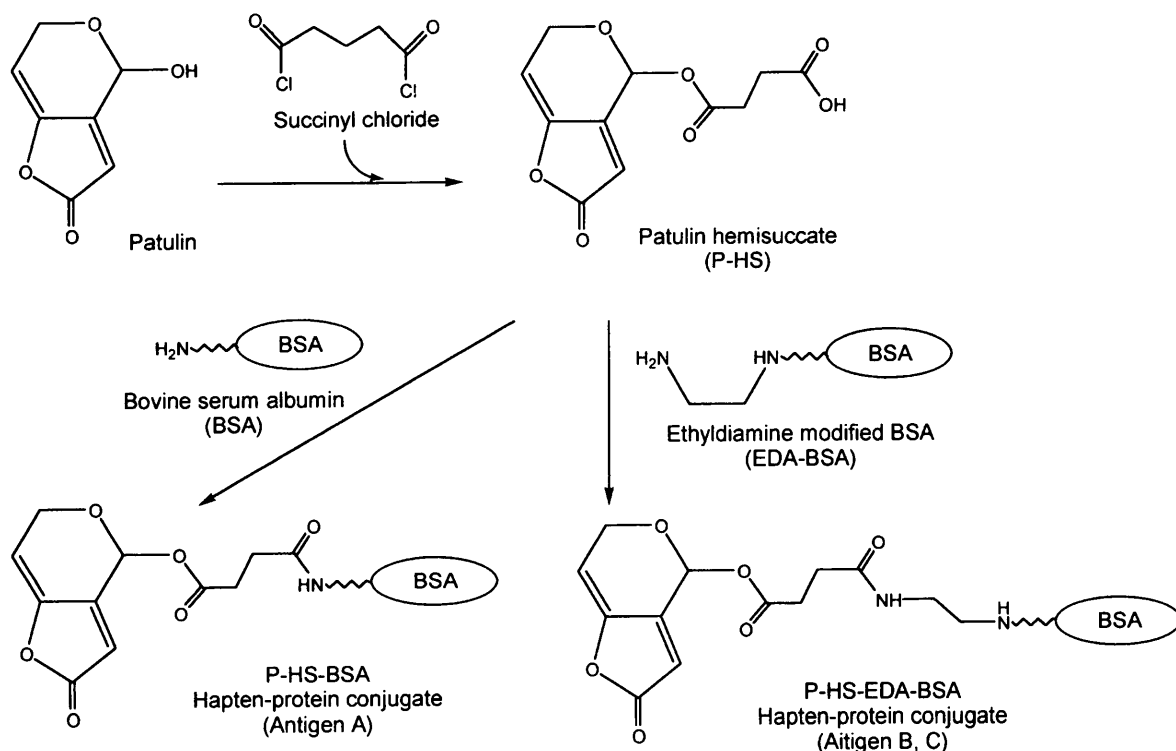

Figure 1. Preparation of the hapten patulin hemisuccinate (P-HS) and the haptan-protein conjugates (P-HS-BSA and P-HS-EDA-BSA). Antigens A and B were synthesized using a water-soluble carbodiimide method and antigen C using a mixed anhydride method.

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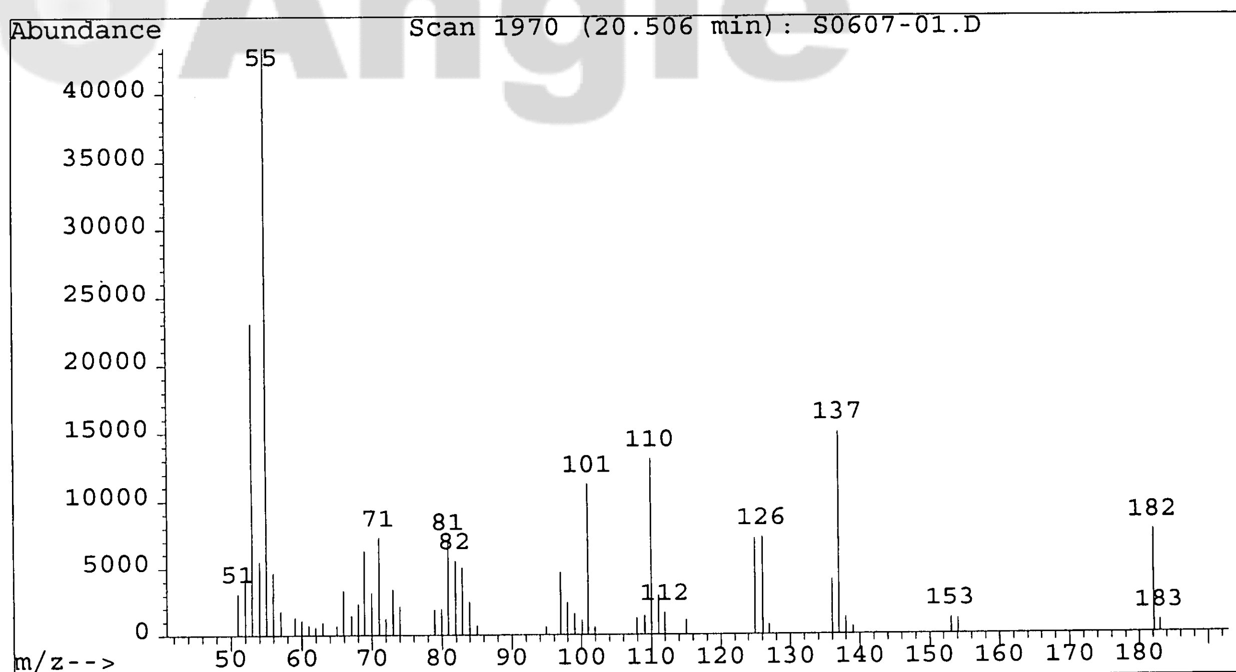

Figure 2. Mass spectrometry MS(EI) showing analysis of patulin hemisuccinate (P-HS).

7.4), gelatin and alkayl phosphatase (AP) substrate was obtained from the Sigma Chemical Co. (St. Louis, Mo). Isobutyl chloroformate and triethylamine were obtained from the TCI Chemical Co. (Tokyo, Japan). 4-N,N-dimethylaminopyridine (DMAP) was obtained from Lancaster (Morecambe, England). Complete and incomplete Freund's adjuvant was obtained from Difco Laboratories (Detroit, Mich.). BALB/c female mice were obtained from the National Animal Institute (Taipei, Taiwan). Tween 20, mouse IgG1 standard and goat anti-mouse IgG-AP conjugate were obtained from the Pierce Chemical Co. (Rockford, IL).

Patulin was confirmed using a Bruker DPX200 spectrometer (NMR). Electron impact mass spectrum was determined using a Hewlett-Packard GC-MS (5890 GC and 5971 mass spectrometer) instrument. The plates used for preparative thin layer chromatography (PTLC) were silica gel (Merck Kieselgel 60 HF_{254}). OD_{405} of ELISA was read with a BioRad 2500 micro plate reader.

II. *Preparation of Hapten-Protein Conjugates*

(I)(S)-*4-hydroxylsuccinoxyfuro[3,2-c]pyran-2-(4H,6H)-one(patulin hemisuccinate, P-HS)*

Patulin (200 mg, 3.25 mmol) and succinyl chloride (1 ml) were dissolved in dry tetrahydrofuran (20 ml), and then 4-N,N-dimethylaminopyridine (1 g) was added. The mixture was stirred for 1 hr, and the solvent was removed under reduced pressure. The residue was partitioned between chloroform (100 ml × 3) and hydrochloric acid (1M, 30 ml). The collected organic layer was dried, evaporated and purified by PTLC (5:3:2 hexane-ethyl acetate-ethanol) to make the hapten patulin hemisuccinate (P-HS, 145 mg, 32%, Rf 0.45) as an oily compound.

(II) *Modification of BSA to EDA-BSA*

BSA was modified with ethylenediamine using the method described by Chu *et al.* (1982) to increase the efficiency of patulin conjugation to the protein carriers. A aqueous EDA (10%, 1 ml) was added to a solution(15 ml) of BSA (300 mg) and EDPC (400 mg) in 15 ml of water. The mixture was adjusted to pH 5.5 by adding hydrochlororic acid (1 N) and was stirred for 2 hr. Additional EDPC (200 mg) was added to the mix-

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ture and the pH was adjusted to 5.5 again. The mixture was allowed to settle overnight and dialyzed against distilled water (1 l) for 3 days with the water changed daily. Then the proteins (EDA-BSA) were lyophilized to dryness.

(III) *Conjugation of Antigen A (P-HS-BSA) and P-HS-OVA*

The water-soluble carbodiimide method was applied for the conjugation of P-HS to BSA. A mixture of P-HS (ca. 10 mg), 20% aqueous ethanol (10 ml), EDPC (100 mg) and BSA (10 mg) was stirred at room temperature for 30 min and then more EDPC (300 mg) was added. The mixture was allowed to settle overnight and dialyzed against distilled water (1 l) for 3 days with the water changed daily. The modified proteins (P-HS-BSA) were lyophilized to dryness. OVA

Table 1. The indirect ELISA titers of diluted antiascites for the six mice

Mice	Antigen injected	Methods of conjugation	Titer
A_1	Antigen A	Water-soluable	16,384
A_2	(P-HS-BSA)	carbodiimide method	16,384
B_1	Antigen B	Water-soluable	8,192
B_2	(P-HS-EDA-BSA)	carbodiimide method	16,384
C_1	Antigen C	Mixed anhydrid method	4,096
C_2	(P-HS-EDA-BSA)		2,048

P-HS-BSA: Patulin hemisuccinate - BSA conjugate; P-HS-EDA-BSA: Patulin hemisuccinate - ethyldiamine modified BSA conjugate.
All experiments were performed in duplicate.

Table 2. The results (OD_{405}) of indirect ELISA titers against different antigens[a]

Antigen coated	Mouse labeled						
	Naive[b]	A_1	A_2	B_1	B_2	C_1	C_2
Patulin	0.018	0.082	0.090	0.028	0.174	0.018	0.022
Patulin hemisuccinate	0.012	0.068	0.062	0.030	0.129	0.018	0.024
P-HS-BSA	0.021	1.851	1.550	0.811	1.545	0.578	0.677
P-HS-EDA-BSA[c]	0.020	1.237	1.108	0.364	1.184	0.277	0.328
P-HS-EDA-BSA[d]	0.028	1.197	1.124	0.331	1.012	0.208	0.294
P-HS-OVA	0.021	0.356	0.268	0.155	0.441	0.087	0.102
OVA	0.024	0.014	0.022	0.022	0.020	0.019	0.019
BSA	0.020	0.033	0.032	0.022	0.043	0.018	0.020
Concentration of coated mouse IgG STD (ng / ml)		0	12.3	37.0	111	333	1000
$O.D._{405}$ of mouse IgG STD		0	0.030	0.067	0.206	0.549	0.875

[a]: Antiascites were 1:100 diluted.
[b]: Mouse was not immunized.
[c]: As antigen B (P-HS-EDA-BSA), which was synthesized using a water-soluable carbodiimide method.
[d]: As antigen C (P-HS-EDA-BSA), which was synthesized using a mixed anhydrid method. All experiments were performed in triplicate and every well was averaged.Relative standard deviation (RSD) was under 10% for all experiments. P-HS-BSA: Patulin hemisuccinate - BSA conjugate; P-HS-EDA-BSA: Patulin hemisuccinate - ethyldiamine modified BSA conjugate.

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instead of BSA was applied for the production of P-HS-OVA using the same method.

(IV) *Conjugation of Antigen B (P-HS-EDA-BSA)*

The water-soluble carbodiimide method was applied for the conjugation of P-HS to EDA-BSA. A mixture of P-HS (ca. 10 mg), ethanol (10 ml), EDPC (100 mg) and protein (EDA-BSA, 10 mg) was stirred at room temperature for 30 min and then more EDPC (300 mg) was added. The mixture was kept overnight and dialyzed against distilled water (1 l) for 3 days with the water changed daily. The modified protein conjugate (P-HS-EDA-BSA) was lyophilized to dryness.

(V) *Conjugation of Antigen C (P-HS-EDA-BSA)*

The mixed anhydride method was applied for the conjugation of P-HS to EDA-BSA. P-HS (ca. 10 mg), triethylamine (10 μl) and isobutyl chloroformate (10 μl) were dissolved in THF (5 ml). The mixture was stirred at room temperature for 30 min and then slowly added to EDA-BSA (25 mg in 15 ml water) and pyridine (8 ml) mixture. The mixture was kept overnight and dialyzed against distilled water (1 l) for 3 days, with the water changed daily. The modified protein conjugate (P-HS-EDA-BSA) was lyophilized to dryness.

III. *Production of Polyclonal Antibody*

(I) *Immunization*

Healthy BALB/c female mice (6-8 weeks old) were immunized by injection of immunogen emulsion consisting of equal volumes of antigen A, B and C and complete Freund's adjuvant in 300-400 ml doses. Injections were performed according to the following schedules at two-week intervals: first, intraperitoneal and intramuscular injection; second, intraperitoneal and subcutaneous injection; third, intraperitoneal injection. A dose of each immunogen was 200 μg / mice. Seven days after last injection, mice were bled by cardiac puncture. The antiascites were separated by means of centrifugation of the ascitic fluid at 3,000 rpm / 5 min.

(II) *Antibody Titeration*

Antibody titer and specificity were determined using an indirect ELISA. Wells of 96-well ELISA microtiter plates (Nunc plate 2-69620; Nunc, Roskilde, Denmark) were coated with the P-HS-BSA conjugate (100 μl, 1 μg/ml diluted with 0.1 M pH 8.2 bicarbonate buffer) and kept at 4°C overnight. After rinsing 4 times with 0.02% (v/v) Tween 20 in PBS (300 μl, PBS-Tween), the plates were blocked with gelatin (150 μl, 0.5 μg/ml in PBS) at 4°C overnight and then rinsed 4 times with PBS-Tween again. Serially diluted (1:1024 up to 1:32768) mouse antiascites (50 μl) were added and incubated at 37°C for 2 hr. Wells of serially diluted preimmune serum were used as control. Unbound antibody was removed by rinsing 4 times with PBS-Tween. Goat anti-mouse IgG-AP (50 μl, 1:1000 diluted in PBS) was added to each well, and then the plates were incubated 37°C for 2 hr and rinsed 4 times with PBS-Tween. The AP substrate (50 μl, 1:1000 diluted in PBS) was added to each well immediately. The OD_{405} of each well was accessed, and the end-point titer of each antiascite was arbitrarily designed as the maximum dilution that yielded twice or more OD_{405} as the same dilution nonimmune control serum.

(III) *Specificity of Antibodies by Indirect ELISA*

A specificity test was carried out using an indirect ELISA. Antigens including patulin, P-HS, P-HS-BSA, P-HS-EDA-BSA, P-HS-OVA, BSA and OVA (all diluted with bicarbonate coating buffer), were added to the wells. The last row of the plates was coated with serially diluted mouse IgG1 standards (from 0 μg/ml to 1 μg/ml). The plates were incubated, rinsed and blocked in titeration as described previously. Antiascites obtained from immunized mouse were diluted (1:100 and 1:1000) and added (50 μl) to each assigned wells except the well coated with mouse IgG standard. After incubation and rinsing, goat anti-mouse IgG-AP (50 μl, 1:1000 diluted) was added to each well, and the plates were incubated and rinsed again. The AP substrate was added to each well,

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and the OD_{405} of each well was accessed.

RESULTS AND DISCUSSION

I. *Preparation of Antigen*

Considering the need for a certain amount of patulin standard, which is costly and unstable during the production of antibody, a complementary synthesis of patulin from arabinose was performed using the methods of Seijas *et al.*[10] and Bennett *et al.*[11] with modifications. An 18% overall yield was obtained and the spectroscopic data were identical to those described in the literature. Since a low molecular weight, such as that of patulin, is not ordinarily antigenic, it is necessary to increase the immunogenicity by linking patulin to carrier protein covalently[2, 9]. For this purpose, the hapten, patulin hemisuccinate (P-HS), was synthesized from succinyl dichloride and patulin, and the yield was 32% (Figure 1). This hapten P-HS was confirmed by mass spectrometry. MS(EI), m/z (%): 55 (100), 71(17), 101 (26), 110 (30), 126 (17), 137 (35), 153 (3), 154 (3), 182 (18) (Figure 2). The additional succinyl chain of P-HS was designed to separate the patulin moiety from the protein and to provide a carboxyl group for hapten-protein conjugation.

Since BSA is an acidic protein, the efficiency for conjugation of P-HS containing a carboxylic group to this protein may be hindered by the availability of the amino groups and also by polymerization between the protein itself[12]. To overcome such problems, two coupling methods including water-soluble carbodiimide method (antigen A as P-HS-BSA and antigen B as P-HS-EDA-BSA) as well as mixed anhydride method (antigen C as P-HS-EDA-BSA) were tried. A modified BSA (EDA-BSA) in which some of the carboxylic groups were blocked by EDA was also prepared for conjugation. However, the degree of conjugation was not successfully determinated because the uv absorption spectra of the patulin (λmax, 276), P-HS (λmax, 282), BSA (λmax, 270), EDA-BSA (λmax, 266) and P-HS-EDA-BSA (λmax, 280) were too close. Proteins were quantified using a BCA protein assay kit (the Pierce Chemical Co, Rockford, IL) before immunization.

II. *Antibody Titers and Specificity*

The antibody titers of the serially diluted antiascites from six mice were 16384, 16384, 8192, 16384, 4096 and 2048, respectively (Table 1). Antibody titers obtained from the mice labeled B_1, C_1 and C_2 were lower than others. Table 2 showed the intensities of OD_{405} obtained through a non-competitive indirect ELISA. Six antigens for the detection of the specificity of antibodies were coated on the plate before ELISA. The IgG concentrations were generally evaluated by referring the OD_{405} intensities to the response curve, which was obtained using the OD_{405} reading vs. the concentration of serially diluted mouse IgG standards in the same test. The titer and specificity tests both showed that mice labeled A_1, A_2 and B_2 were better immunized than others. The low titers of antibodies against BSA showed that the moeity of the protein conjugates was no longer similar to the original BSA. It was very interesting that P-HS-BSA was better recognized by the antiascites of all six mice including the mice (B_1, B_2, C_1 and C_2) which were immunized by using P-HS-EDA-BSA. This could be due to the incomplete reaction of P-HS conjugated to EDA-BSA, which made the surface of the conjugate P-HS-EDA-BSA nonuniform and also decreased the immunization as well as the affinity toward antibodies as determined by ELISA. This result did not agree with the conclusion drawn by Chu *et al.* (1982)[12]. The immunogenicities of the conjugate P-HS-EDA-BSA that was synthesized using a mixed anhydride and a water-soluble carbodiimide method showed no difference.

Patulin and the hapten P-HS were only slightly recognized by the antiascites obtained from mice A_1, A_2 and B_1 since the OD_{405} were significantly higher than the control ELISAs of BSA and OVA (Table 2). Perhaps the patulin and hapten P-HS were not sufficiently bounded to the plate thus causing the poor titer against patulin and P-HS. The titer of antibodies against patulin decreased

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after purification by means of ammonium precipitation. The antiascites obtained from naive mice showed no response to all immobilized antigens. Positive response of the antiascites to the immobilized P-HS-OVA antigen showed that the antibodies produced against the P-HS-BSA or P-HSEDA-BSA complex also recognized P-HS-OVA.

Weiss and McElroy (1993) first reported the production of polyclonal antibodies against the mycotoxin derivative, patulin hemiglutarate[(2)]. However, their method for purifying the hapten patulin hemiglutarate and confirming the structure was not given and the specific antibodies against patulin alone were not clearly demonstrated. In this study, P-HS used as hapten conjugated to carrier protein was synthesized because of the higher yield compared to patulin hemiglutarate as determined in the preliminary study[(4)]. The affinity of antibody to patulin alone was also demonstrated directly and clearly. Although the obtained antibodies were not good enough for quantitative analysis of patulin, the results showed the possibility of getting the monoclonal antibody against patulin by screening hybridomas from mice. An effort to do so is on going in the same laboratory.

ACKNOWLEDGEMENT

We extend our sincere thanks to Dr. H. G. Su, Dr. M. L. Wu (Department of Plant Pathology, NTU) and Dr. K. Y. Hsieh (Department of Immunology, NTU) for their constructive suggestions regarding mouse immunization and ELISA.

REFERENCES

1. Harrison, M. A. 1987. Presence and stability of patulin in apple products: A review. J. Food Safety 9: 147-153.
2. McElroy, L. J. and Weiss, C. M. 1993. The production of polyclonal antibodies against the mycotoxin derivative patulin hemiglutarate. Can. J. Microbiol. 39: 861-863.
3. The Association of Official American Chemists. 1984. Official Methods of Analysis. 14th ed. pp. 496-497. AOAC, Arlington, VA, U.S.A.
4. Sheu, F. and Shyu, Y. T. 1995. Studies on the methodology of patulin analysis in apple juice - comparison of the thin layer chromatography and gas chromatography. J. Chinese Soc. Horticul. Sci. 41: 127-135.
5. Brause, A. R., Trucksess, M. W., Thomas, F. S. and Page, S. W. 1996. Determination of patulin in apple juice by liquid chromatography: Collaborative study. J. Assoc. Off. Anal. Chem. 79: 451-455.
6. Azcona-Olivera, J. I., Abouzied, M.M., Plattner, R. D. and Pestka, J. J. 1992. Production of monoclonal antibodies to the mycotoxins fumonisins B_1, B_2 and B_3. J. Agric. Food Chem. 40: 531-534.
7. Huang, X. and Chu, F.S. 1993. Production and characterization of monoclonal and polyclonal antibodies against the mycotoxin cyclopiazonic acid. J. Agric. Food Chem. 41: 329-333.
8. Chu, F. S. and Ueno, I. 1977. Production of antibody against aflatoxin B_1. Appl. Environ. Microbiol. 33: 1125-1128.
9. Erlanger, B. F. 1980. The preparation of antigenic hapten - carrier conjugates: A survey. Acad. Press. Methods in Enzymology 70: 85-104.
10. Seijas, J. A., Tato, P. V., Estevez, R., Castedo, L. and Riguera, R. 1989. New total synthesis of patulin. Heterocycles 29: 181-184.
11. Bennett, M., Gill, G. B., Pattenden, G. Shuker, A.J. and Stapleton, A. 1991. Ylidenebutenolide mycotoxin. Concise syntheses of patulin and neopatulin from carbohydrate precursors. J. Chem. Soc. Perkin Trans. 1: 929-937.
12. Chu, F. S., Lau, H. P., Fan, T. S. and Zhang, G. S. 1982. Ethylenediamine modified bovine serum albumin as protein carrier in the production of antibody against mycotoxins. J. Immunol. Methods. 55: 73-78.

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Journal of Food and Drug Analysis. 1999. 7(1)

抗棒麴黴素衍生物抗體生產及抗原之合成

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摘　要

棒麴黴素(patulin)是園產品中最重要的眞菌毒素。本研究合成丁醯棒麴黴素(patulin hemisuccinate)並將其耦合至小牛血清蛋白上，以此複合抗原(patulin-hemisuccinate-BSA)免疫白鼠，再由白鼠體內抽取抗腹水(antiascite)並得到多株抗體。經由間接非競爭性酵素連結免疫分析法測定血清的效價及專一性，結果顯示來自六隻中三隻白鼠的抗體效價較高，且對複合抗原具強烈反應。抗體對單獨棒麴黴素的反應較微弱，但顯示了利用此技術發展抗棒麴黴素單株抗體的潛力。

關鍵詞：棒麴黴素衍生物，多株抗體。