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Purification and relationship with gastric disease of a 130 kDa (CagA) protein of *Helicobacter pylori* *

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Abstract: Objective: The aims of this research were to purify and identify the 130 kDa (CagA) protein of *H. pylori* clinical isolate HP97002 and evaluate the relationships between the purified 130 kDa (CagA) protein and gastric diseases. Methods: The procedure for isolating the protein included 6 mol/L guanidine extract, size exclusion chromatography and elusion from gel. Sera of 68 patients with gastric diseases (44 with chronic gastritis, 15 with atrophic gastritis, 7 with peptic ulcer disease, 2 with gastric cancer) were obtained, and the serological response to CagA was studied by Western-blot using the purified protein. Results: The purified protein was 130 kDa and preserved good antigenicity and revealed basic isoelectric point about of 8.1. Among 68 sera, 43 sera could recognize the purified protein associated with chronic gastritis 47.7% (21/44), atrophic gastritis 86.7% (13/15), peptic ulcer disease 100% (7/7), gastric cancer 100% (2/2). Compared with each other, the difference was significant ($\chi^2 = 13.327$, $P = 0.004$), and 130 kDa (CagA) protein was associated with severe gastric diseases ($r_s = 0.442$, $P = 0.001$). Conclusion: The 130 kDa (CagA) protein was associated with severe gastric diseases.

Key words: *Helicobacter pylori*, CagA, Elution from gel, Western-blot
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INTRODUCTION

Helicobacter pylori infection had been recognized as an important causative factor in chronic gastritis and peptic ulcer disease (PUD) and has been considered to be a risk factor for gastric cancer and mucosa-associated lymphoid tissue-type lymphomas of the stomach (gastric MALTomas) (Blaser, 1992; Blum, 1996). However, although essentially all infected persons develop gastritis, a considerable number remain asymptomatic whereas a minority of persons develop severe diseases, such as ulcer, atrophic gastritis, or adenocarcinoma (Crabtree, 1996). One explanation for different clinical outcomes is the diversity of *H. pylori* strains (Blaser, 1997; Megraud, 1997). In comparisons between strains, however only cagA gene (cytotoxin-associated gene A) and its product, CagA protein has been shown to correlate with the severity of disease (Lee, 1996). Infection with a cagA-positive strain is both highly associated with peptic ulcer disease and with the risk of developing intestinal metaplasia, atrophic gastritis, and adenocarcinoma of the stomach (Crabtree et al., 1991; Cover et al., 1995). This conclusion is supported by the results of mouse model. The sonicates of cagA-positive *H. pylori* caused murine mucosal injuries like human's (for example, gastric epithelial cell vacuolation and mucosal tissue erosion, necrosis and ulcer), whereas cagA-negative *H. pylori* could not induce gastric lesions (Telford et al., 1994).

For these reasons, we sought to purify the 130 kDa (CagA) protein of clinical isolate HP97002 by principles and methods of membrane protein and evaluated the relationship between the purified CagA protein and gastric diseases. In addition, the production of purified 130 kDa (CagA) protein may be of value for diagnostic and vaccine development purposes.

MATERIALS AND METHODS

Bacterial strains and growth conditions

H. pylori 97002, a previously described ca-

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gA-postive strain (Ye et al., 1999) isolated from a patient with peptic ulcer was used as the source for CagA protein purification. *H. pylori* 97002 was cultured for 48 hours at 37℃ in solid ECY media (Fang, 1993) in a microaerobic atmosphere containing 5% oxygen, 10% carbon dioxide, 85% nitrogen. Cells from plates were harvested in 0.01 mol/L phosphate-buffered saline (PBS), centrifuged twice at 5000 × g for 20 min, and the pellet was stored at −20℃ until used.

Purification of 130 kDa (CagA) protein

Frozen cell pellets were thawed, suspended in PBS containing 0.2 mmol/L phenylmethyl sulfonyl fluoride, and adjusted OD_{450} of 0.6. The suspension was treated with guanidine at a final concentration of 6 mol/L for 60 min at room temperature. After centrifugation at 10000 × g for 20 min, the supernatant was removed and dialyzed 48 hours against a buffer containing 0.05 mol/L pH7.4 Tris-HCl. The supernatant was chromatographed on a column (1.6 × 100cm) of Sephadex G-200 (Pharmacia), equilibrated, and eluted with the same buffer of 0.05 mol/L pH7.4 Tris-HCl at flow rate of 0.14 ml/min. Column eluates were monitored for UV absorbance at 280 nm. The flow-through contained the 130 kDa protein was further purified by a modified elution method from SDS-polyacrylamide gel according to Bridgen (Bridgen, 1976). Briefly the preparative 7% SDS-polyacrylamide gel electrophoresis was run according to (Leammli, 1970) at 120 V. Per gel 180 μg of proteins (the flow-through proteins) was loaded. On the same gel one lane was loaded with 15 μg of molecular marker proteins including myosin (212 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), catalase (57.5 kDa) and aldolase (40.0 kDa). After electrophoresis the lane was separated, stained with Coomassie Blue, destained, and was used to localize the protein of interest in the preparative gel. This protein band was cut out, crushed, and extracted for 24 hours with buffer of 0.05 mol/L NH_4HCO_3, 0.05% SDS. After the gel suspension was centrifuged, the supernatant was dialyzed and subsequently concentrated by lyophilization.

Sera

A mixture of sera from five gastric ulcer patients with *H. pylori* infection was used as *H. pylori* positive serum. *H. pylori* negative serum was used as control. Rabbit antiserum was obtained from rabbits immunized with NCTC11637 strain.

Western-blot analysis

Western-blot was performed according to Bumatte (Bumatte, 1981). Following separation by SDS-PAGE, proteins were transferred to nitrocellulose paper by electroblotting for 20 hours at 10V. At room temperature after nonspecific binding was blocked with mild-borate buffer, the nitrocellulose papers were incubated for 2 hours with 1:50 dilutions of human sera. After being washed and incubated with 1:50 dilutions of horseradish peroxidase-conjugated staphylococcal protein A (HRP-SPA) for 2 h, the nitrocellulose papers were stained with Diaminobenzidine (DAB).

Isoelectric focusing

Isoelectric focusing was performed with 2% Ampholine (pH range 3 to 10). Proteins were focused for 6 h at 180 V with anode buffer (0.01 mol/L H_3PO_4) and cathode buffer (0.02 mol/L NaOH).

Patients

A total of 68 patients examined and underwent of endoscopy as well as th antral biopsies. The patients had received neither antimicrobial nor antiacid therapies during the previous 3 weeks. The biopsies were processed for culture of *H. pylori* and for histology and rapid urease test. Sera were collected the day of the endoscopy. Analysis of data was done by using Chi-square(χ^2) test and Spearman's correlation under Windows SPSS 8.0.

RESULTS

Purification of 130 kDa (CagA) protein:

The purification of the 130 kDa (CagA) protein of *H. pylori* involved 6 mol/L guanidine extract, size exclusive chromatography and elution from gel. The guanidine extract was chromatographed on Sephadex G-200, and the protein was detected in the first pead that eluted from the column (Fig. 1). This purification scheme resulted in a single protein band with an apparent $Mr = 130$ kDa on SDS-PAGE (Fig. 2).

Western-blot showed this protein preserved good antigenicity (Fig. 3). The isoelectric focusing analysis of the purified protein revealed a basic isoelectric point of 8.1.

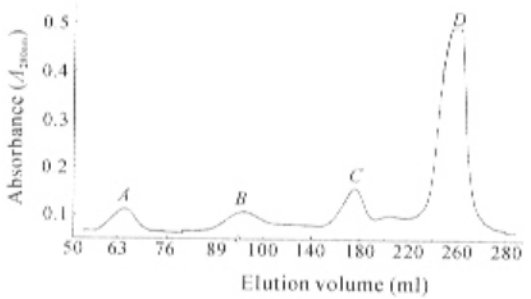

Fig.1 Isolation of *H. pylori*97002 guanidine extract with Sephadex G-200 column

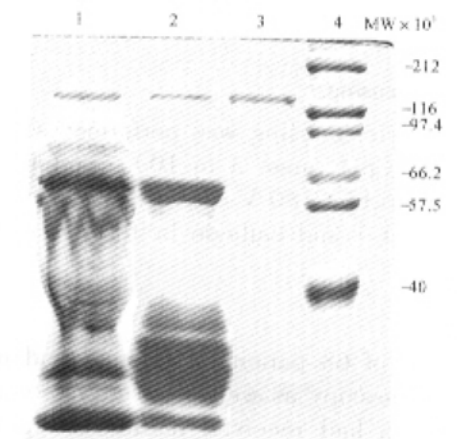

Fig. 2 SDS-PAGE analysis of purified *H. pylori* protein, the separating gel contained 10% acrylamide

Lane 1: guanidine extract; lane 2: fraction A of Gel Filtration Chromatography; lane 3: purified *H. pylori* protein; lane 4: M.W. Marker

The relationship between the purified 130 kDa (CagA) antigen and gastric diseases:

Among 68 sera, 43 sera could recognize the purified proteins from the patients with chronic gastritis 47.7% (21/44), atrophic gastritis 86.7% (13/15), peptic ulcer disease 100% (7/7), gastric cancer 100% (2/2). Compared with each other, the difference was significant ($\chi^2 = 13.327$, $P = 0.004$), and 130 kDa (CagA) protein was shown to be associated with severe gastric diseases ($r_s = 0.442$, $P = 0.001$) (Fig. 4) (Table 1).

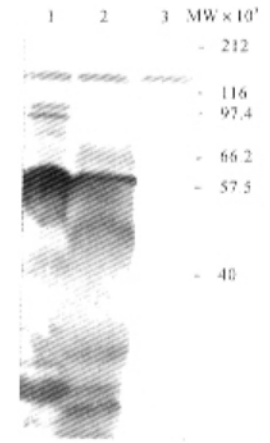

Fig.3 Western-blot analysis of purified *H. pylori* protein

Lane 1: guanidine extract; lane 2: fraction A of Gel Filtration Chromatography; lane 3: purified *H. pylori* protein. The numbers on the right indicate molecular weight in thousands.

Fig.4 Western-blots analysis of sera from patients with gastric disease against the purified 130 kDa (CagA) protein

Lane a: sera of five *H. pylori*-infected patients with gastric ulcer; lane b: serum from a rabbit immunized with NCTC11637 strain; lane c: serum obtained from a *H. pylori*-infected person; lane d-g: sera of patients with gastric diseases.

DISCUSSION

The CagA antigen had been described mostly as present in the culture supernatant of strains grown in the presence of calf serum (Cover et al., 1990). Van der and his colleagues (van der Ende et al., 1996) purified the CagA protein from the culture supernatant with procedure including ammonium sulfate precipitation, hydrophobic interactive chroma-tography, size exclusion chroma tography and anion exchange chromatography. In this

Table 1 The links between prevalence of CagA and *H. pylori* and four gastric diseases

		chronic gastritis ($n=44$)	atrophic gastritis ($n=15$)	peptic ulcer disease ($n=7$)	gastric cancer ($n=2$)
CagA*	+	47.7%(21)	86.7%(13)	100% (7)	100%(2)
HP#	+	72.1%(31)	86.7%(13)	85.7%(6)	50.0%(1)

* $\chi^2=13.327$, $P=0.004$; Spearman's $r_s=0.442$, $P=0.001$

\# $\chi^2=2.407$, $P=0.475$

study, we selected ECY solid medium without supplemental fetal bovine serum, so many nonbacterial proteins were thereby eliminated. The CagA antigen is a hydrophilic, surface-exposed protein estimated to be 120-130 kDa by various authors (Covacci et al., 1993). With 6mol/L guanidine extract, size exclusive chromatography and elution from gel, we succeeded in purifying the 130 kDa (CagA) protein from *H. pylori* cells.

Approximately 60% of *H. pylori* isolates in the Western world possess the cytotoxin-associated gene A (cagA). Importantly, infection with cagA-positive strain is both highly associated with peptic ulcer disease, and the risk of developing intestinal metaplasia, atrophic gastritis, and adenocarcinoma of the stomach (Sozzi et al., 1998). In this study, we analyzed the link between the prevalence of CagA and gastric diseases using Western-blot analysis. Among 68 sera, 43 sera could recognize the purified proteins from the patients of chronic gastritis 47.7%(21/44), atrophic gastritis 86.7% (13/15), peptic ulcer disease 100%(7/7), gastric cancer 100%(2/2). The results of this study suggested that CagA is associated with the development of more serious gastric disease.

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