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Characterization of a *Helicobacter pylori* vaccine candidate by proteome techniques

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Abstract

In a previous two-dimensional (2D) gel electrophoretic study of protein antigens of the gastric pathogen, *Helicobacter pylori* recognized by human sera, one of the highly and consistently reactive antigens, a protein with M_r of approximately 30 000 (Spot 15) seemed to be of special interest because of low yields on N-terminal protein sequencing. This suggested possible N-terminal modification, as the N-terminal sequence analysis of this 30 000 protein (Spot 15) did not provide a definitive match within the *H. pylori* genomic database. This protein was isolated by 2D polyacrylamide gel electrophoresis, evaluated by liquid chromatography–mass spectrometry, and found to consist of two related species of approximately 28 100 and 26 500. In parallel, the proteins within this spot were digested in situ with the endoprotease Lys-C. Analysis of the Lys-C digest by matrix-assisted laser desorption time-of-flight mass spectrometry, peptide mapping, and sequence analysis was conducted. Comparison of the mass and sequence of the Lys-C peptides with those derived from a *H. pylori* genomic library identified an open reading frame of approximately 300 base pairs as the source of the Spot 15 protein. This corresponded to HP0175 in the recently reported *H. pylori* genome sequence, an open reading frame with some homology to *Campylobacter jejeuni* cell binding protein 2. Mass spectral and sequence analysis indicated that Spot 15 was a processed product generated by proteolytic cleavage at both the carboxy and amino termini of the 34 open reading frame precursor.

Keywords: Proteome technique; *Helicobacter pylori* vaccine

1. Introduction

H. pylori is a Gram negative bacterium that chronically infects the gastric mucosa of half or more of all humans worldwide, and that is a major cause of gastritis and peptic ulcer disease and an early risk factor for gastric cancer. DNA typing has established that *H. pylori* is extremely diverse as a species, and it is likely that the varied outcomes of infection reflect differences in bacterial genotype and genetic, physiological, and immunological factors in the human host [1]. These considerations make it particularly important to fully characterize the proteins and other antigens that various *H. pylori* strains produce, and the human responses to them.

Colonization and virulence factors of *H. pylori* are

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just beginning to be identified. Some of the more prominent factors include (i) the flagella, which allow movement in the mucous layer [2]; (ii) the urease complex, which is needed for colonization presumably by maintaining the micro pH environment [3]; (iii) the VacA protein that causes the vacuole formation in eukaryotic epithelial cells and is disease associated epidemiologically [4]; and (iv) the cag pathogenicity island which is also disease associated, and may help elicit severe damaging inflammatory responses in infected patients [5]. Many other proteins, or likely gene products identified in part by scanning *H. pylori* genomic DNA sequences have been isolated. Most recently the complete genome sequence of *H. pylori* has been made available [6]. While DNA sequences provide an excellent database for determination of proteins of potential therapeutic value, many of the putative proteins or rather 'proteomes' isolated from *H. pylori* DNA sequence information have no known function.

In a previous study we evaluated proteins from several strains of *H. pylori* using pooled sera from *H. pylori*-infected and *H. pylori*-free donors [7]. At least 30 antigens that are recognized by the *H. pylori* infected serum pool were identified by two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Based on N-terminal sequence analysis, 14 of the 30 proteins isolated that reacted with disease positive human sera were new, and unrelated to any previously known *H. pylori* proteins, whereas most others matched proteins known from previous studies of *H. pylori* gene products.

Here, we report that isolation and characterization of a particular highly conserved but previously unknown *H. pylori* antigen. The application of proteomics to the isolation and characterization of other antigens should significantly enhance our understanding of *H. pylori* and its interaction with its human host.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions for H. pylori

Initial 2D characterization and isolation of *H. pylori* antigens was performed using the type strain ATCC 43504 (also called NCTC11637) which was isolated from a peptic ulcer patient at Royal Perth Hospital, Australia. Other strains used for comparative purposes were from the laboratory collection of Dr. D.E. Berg. All *H. pylori* strains were cultured on trypticase soy agar (TSA) BBL media supplemented with 10% defibrinated sheep's blood (Quad 5, Helena, MT, USA) in microaerobic chambers using the Pack-Campylo system (Mitsubishi Gas Chemical America, USA). Cells harvested from TSA blood agar plates were washed with phosphate buffered saline (PBS) and lysed by sonication.

2.2. Two-dimensional gel electrophoresis (pH 8–13)

Two-dimensional electrophoresis adapted for resolution of basic proteins was performed according to the method of O'Farrell [8] as follows: proteins were separated by nonequilibrium pH gradient electrophoresis in gels containing 4% acrylamide (acrylamide–bis acrylamide ratio: 37:5, w/v) 9.5 *M* urea, 3.6% CHAPS in glass tubes of I.D. 2.0 mm using 1.5% pH 3.5–10 and 0.25% pH 9–11 ampholines (Pharmacia Biotechnology, Piscataway, NJ, USA) was carried out at 140 V for 12 h. Purified tropomyosin, lower spot M_r 33 000 and p*I* 5.2, and purified lysozyme M_r 14 000 and p*I* 10.5–11 (Merck Index) were added to the samples as internal p*I* markers. After equilibration for 10 min in buffer (10% glycerol, 50 m*M* DTT, 2.3% SDS and 62.5 m*M* Tris, pH 6.8) the tube gel was sealed to the top of the stacking gel which was placed on top of a 10% acrylamide slab gel (0.75 mm thick) and SDS slab gel electrophoresis was carried out for 4 h at 12.5 mA/gel. The slab gels were fixed in a solution of acetic acid–methanol (10:50, v/v) overnight. The following proteins were added as molecular mass standards to the agarose which sealed the tube gel to the slab gel: myosin (220 000), phosphorylase A (94 000), catalase (60 000), actin (43 000), carbonic anhydrase (29 000) and lysozyme (14 000). These standards appear as horizontal lines on the silver stained 10% acrylamide slab. The silver stained gel was dried between sheets of cellophane paper with the acid edge to the left. Silver staining [9] was only performed if gels were not subjected to Western blotting.

2.3. Western blot

Following slab gel electrophoresis, the gel was transferred to transfer buffer (12.5 m*M* Tris, pH 8.8, 86 m*M* glycine, 10% methanol), transblotted onto polyvinylidenedifluoride (PVDF) paper overnight at 200 mA (approximately 50 V/gel). The blot was blocked for 2 h in 2% bovine serum albumin (BSA) in Tween–Tris-buffered saline (TTBS), rinsed in TTBS, incubated in primary antibody (positive serum pool described below) diluted 1:2500 in 1% BSA–TTBS for 2 h, rinsed in TTBS and placed in secondary antibody (antihuman IgG HRP, 1:5000 diluted in TTBS) for 1 h. The blot was rinsed with TTBS, treated with ECL (Amersham-Pharmacia Piscataway, NJ, USA) and exposed to X-ray film.

2.4. Serum pools

The positive serum pool was derived from pooled sera obtained from 14 patients identified by endoscopy as *H. pylori* positive. Biopsied material from endoscopy was confirmed to contain *H. pylori* by culture and standard histological staining techniques. The negative serum pool was derived from 20 volunteers with no previous history of gastric disorder.

2.5. In gel lys-C digestion

Two dimensional gel fragments and a control gel fragment were digested in situ with Lys-C protease followed by reductive alkylation of the Cys residues. The proteolytic fragments were then extracted and HPLC mapped [10]. The gel fragments were pretreated with 500 μl of 50% acetonitrile (ACN), 200 m*M* ammonium carbonate, pH 8.9, to partially dehydrate and destain the gel fragments. A 200-μl volume of a solution containing 250 ng of Lys-C protease (*Achromobacter lyticus* lysyl endopeptidase, Boehringer Mannheim, Indianapolis, IN, USA), 0.1% hydrogenated Triton X-100, 200 m*M* ammonium carbonate, pH 8.9, was added to the gel fragments. The rehydrated gel fragments were incubated at 40°C for approximately 24 h. The proteolytic fragments were then extracted into 30 μl of a solution of 60% ACN, 0.1% trifluoroacetic acid (TFA).

2.6. N-terminal sequencing

Sequencing was carried out on a Hewlett-Packard G1005A N-terminal sequencer using Hewlett-Packard reagents. The employed technology gave a high repetitive yield (typically 93–98%) with a detection limit of approximately 100–200 fmol. All sequences were compared to data in the NCBI, TIGR, Swiss Protein, and our own *H. pylori* database using BLAST and BLITZ programs.

2.7. Capillary reversed-phase HPLC

Analysis of Spot 15 Lys-C digests Capillary LC was performed on the Lys-C digested Spot 15 protein using an ABI syringe pump model 410 connected to a Vydac C_{18} fused capillary 150×1 mm reversed-phase column at a flow-rate of 50 μl/min. The starting buffer (buffer A) was 0.1% TFA in water with the gradient buffer (buffer B) of 0.1% TFA in acetonitrile–water (9:1) v/v. Buffer A (100%) was run for 5 min followed by a linear gradient of 0 to 100% buffer B over 55 min.

2.8. Liquid chromatography–mass spectrometry

LC–MS procedures were performed as described previously [10]. Spot 15 protein and Lys-C digests were chromatographed on a Vydac C_{18} reverse phase capillary column as described above. A Carlo Erba Phoenix 20 CU pump was used to deliver methoxyethanol–isopropanol (1:1, v/v) at 50 μl/min which was combined with the column eluent in a postcolumn mixing chamber. An in-line flow splitter was used to restrict flow to the mass spectrometer to approximately 10 μl/min. Detection was performed immediately following elution from the column at 214 nm using an ABI 759A variable wavelength detector. Mass spectrometric detection was achieved following postcolumn solvent addition and flow splitting by a VG BioQ triple quadrupole mass spectrometer. Spectra were recorded in the positive and negative ion mode using electrospray ionization.

2.9. Laser desorption time-of-flight mass spectroscopy of lys-C digests

Aliquots of peptides via reversed-phase HPLC were added on top of a 1 μl α-cyano-4-hydroxy-

cinnamic acid (α-CHCA) matrix solution that was spotted onto a new target as previously described [10].

2.10. Preparation of H. pylori genomic clones

H. pylori genomic DNA was prepared as described [11] with minor modifications and partially digested with pancreatic DNAse I (Boehringer Mannheim) [12]. The resulting DNA fragments were resolved by preparative agarose gel electrophoresis (BioRad, Walnut Creek, CA, USA), and DNA fragments in the desired size range (200–2000 base pairs) were excised from the gel, and recovered using the Gene Clean II kit (Bio 101, La Jolla, CA, USA) according to the manufacturer's instructions. To generate blunt ends, the recovered DNA fragments were incubated with *E. coli* Klenow fragment of DNA polymerase at room temperature for 30 min (Promega, Madison, WI, USA). The reaction was stopped by phenol–chloroform extraction, and the resulting DNA fragments were ligated and amplified by sequence independent single primer amplification (SISPA; [13]). The pool of amplified DNAs were digested with *Eco*RI, short oligonucleotides and DNA fragments were removed by gel filtration, and remaining digested DNAs were then ligated into lambda gt11 or lambda ZAPII phage vector arms using T4 DNA ligase (Boehringer Mannheim). The ligated DNA was packaged into λ phage heads by standard procedures using a Gigapak kit, (Stratagene, La Jolla, CA USA), and then plated at various dilutions on strains Y1089 (λgt11) and XL1Blue (λZAPII) on standard X-gal (Stratagene) plates to determine the titers of the DNA phage libraries and percent recombinants. The titers ranged from $5 \cdot 10^5$ to $3 \cdot 10^6$ plaque forming units (PFU)/ml. Phage plaques from these libraries were immunoscreened for the production of antigens recognized by pooled sera from 14 *H. pylori* patients essentially as described [14].

3. Results

3.1. Two-dimensional SDS-PAGE, pH 8–13 and western blotting

Our previous study revealed several unique *H. pylori* encoded proteins whose sequences were not then present in any public database. One prominent spot, Spot 15, reacted strongly with antibodies in the positive serum pool and was evaluated further (see Fig. 1). Spot 15 was extracted and evaluated by ES-MS and determined to consist of two distinct proteins of 28 020 and 26 260 (see Fig. 2), Assuming at this point that the two species might be related to each other (both were Western blot positive), two pmoles of Spot 15 was digested in situ (from an excised polyacrylamide gel slice) with Lys-C to generate internal peptide fragments for sequence characterization. A blank gel slice was analyzed in parallel as a control.

3.2. Lys-C peptide analysis

The resulting peptides were resolved by reversed-phase HPLC (Fig. 3). Out of over 100 potential peaks detected by reversed-phased HPLC analysis, 18 which appeared to be relatively pure based upon UV absorption profiles were selected for sequence analysis and laser desorption mass spectrometry.

3.3. Mass spectrometry and sequence analysis

Thirteen peaks (47, 54, 57, 58, 60, 63, 66, 68, 69,70, 81, 88 and 89) were evaluated by N-terminal sequence analysis and MALDI-TOF MS to determine whether: (i) whether the peak was a peptide or an artifact peak; (ii) the peak contained more than one peptide; and (iii) whether the MS calculated mass was comparable to the predicted mass from protein sequence, and also from DNA sequence analysis of the corresponding gene (see below). Post-translational modifications would be readily detected by this approach.

As a parallel means of identifying new potentially useful antigens for detection of *H. pylori*, a DNA library was prepared in either the expression vector lambda gt11 or ZAPII. Phage that expressed peptides that were immunoreactive with pooled sera were then selected and several clones were found, as expected. These clones were also screened in parallel with anti-cag A antibody by hybridization with gene probes for the following proteins: cagA [15], urease A [16], vac A [4], lpp 20 precursor [17], hpn protein

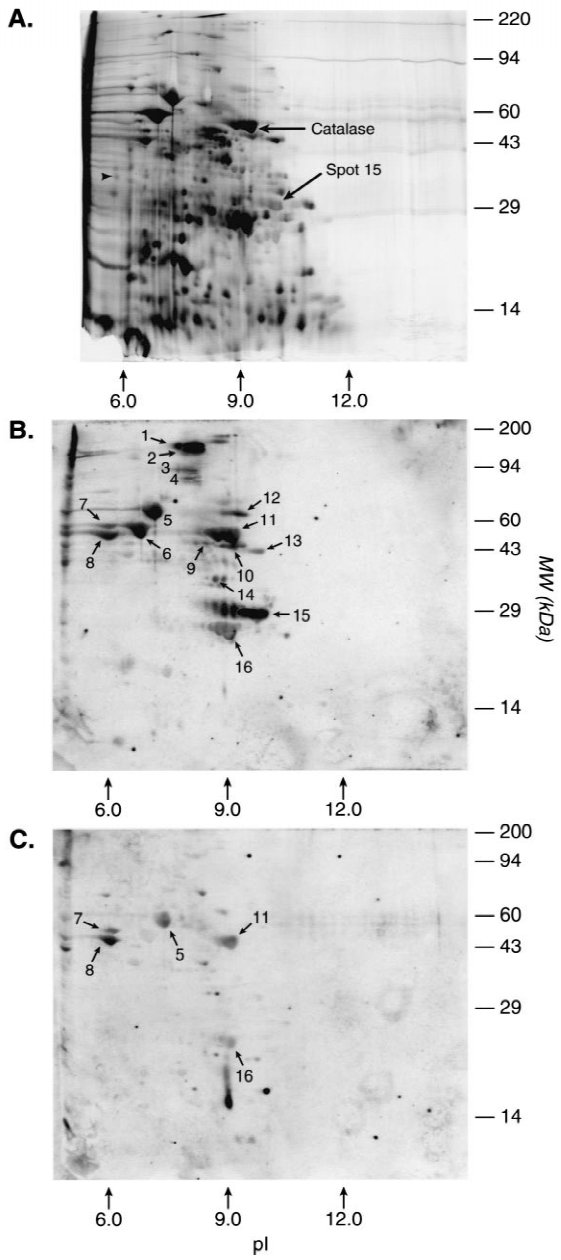

Fig. 1. *Helicobacter pylori* two-dimensional map (nonequilibrium pH gradient electrophoresis pH 8–13) strain 43504 was grown as described in Section 2.1, lysed by sonication and protein concentration determined by Pierce BCA assay (Pierce Chemicals Rockford, IL USA). Protein extract (200 μg) was loaded on the first dimensional gel. (A) Silver-stained gel: tropomyosin (50 ng) was added as an internal IEF standard as described in Section 2.2 (unlabelled arrowhead). Purified lysozyme (M_r 14 000, p*I* 10.5–11.0) was also added as an internal p*I* standard. (B) Western blot using a positive serum pool. (C) Western blot using a negative serum pool.

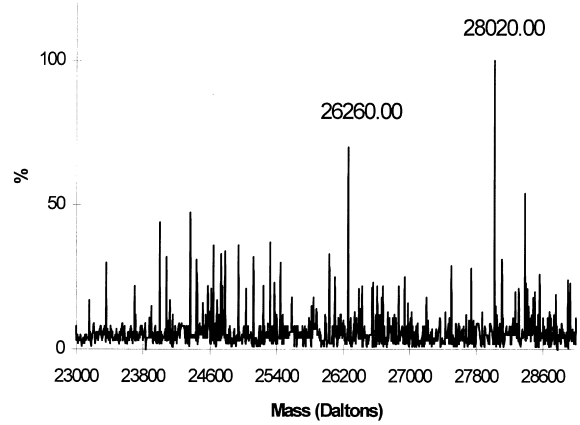

Fig. 2. Electrospray-MS. Deconvoluted MS electrospray mass spectrometry analysis of Spot 15. MS detection was achieved using a VG BioQ triple quadrupole mass spectrometer operating in the positive ion electrospray ionization mode. The major protein components are noted in the figure.

[18], and the 26 000 O'Toole protein [19]. This prescreening eliminated approximately 50% of our clones. Following this prescreen, 462 recombinant clones were identified with the positive serum pool. These clones were separated into 69 gene clusters from which 170 open reading frames were derived.

One of the serum positive clones, Y104B, was found to encode an open reading frame with a predicted amino acid sequence which matched MALDI-TOF Lys-C peptides derived from Spot 15. It was sequenced and the predicted amino acid sequence was compared to data obtained from MS and protein sequencing. A summary of the mass spectral data and sequence analysis is presented in Figs. 4 and 5. Six peaks (54, 58, 60, 68, 70 and 89) appeared to be mixtures that would normally require HPLC repurification prior to individual sequencing. However, the amino acid sequence predicted from the Y104/Spot15 genomic clone made it possible to deduce individual peptide sequences from the mixed amino acid sequence. The N-terminal sequence information from intact Spot 15 suggested that the protein had the amino terminal sequence; PX(H)NA — although the sequencing yields were unexpectedly low which implied that the majority of this peptide preparation was N-terminally blocked. Analysis of the corresponding *H. pylori* clone identified this amino acid sequence at the N-terminus of the 34 000 protein of the Y104B clone. The predicted molecular mass of

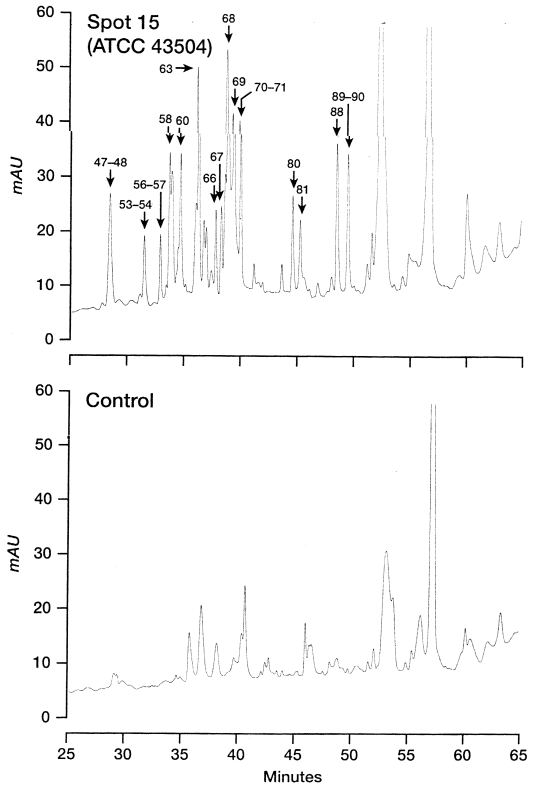

Fig. 3. Reversed-phased microbore HPLC. Capillary reversed-phase HPLC analysis of Lys-C peptides from Spot 15. Numbered peaks correspond to LD–MS results and sequenced peptides from Spot 15 in Figs. 4 and 5. HPLC conditions were as described in Section 2.7. Full scale corresponds to 0.1 absorbance units at 214 nm.

the Lys-C peptide encompassing these residues would be 1390.4.

The molecular mass inferred from MS although about 43 larger than this 1390.4 prediction, suggested that peptide 47 represented the N-terminal Lys-C peptide although it did not yield an interpretable sequence. This discrepancy could be ascribed to acetylation of the amino terminus of the proline residue. Postsource decay (data not shown) confirmed that this peptide (residues 23–35 of the Y104B open reading frame) was the amino terminal peptide of Spot 15. While proteolytic clips at KP junctions followed by acetylation are unusual in most bacterial systems studied, this protein would appear to undergo a specific processing event in *H. pylori*.

Lys-C Peptide	Residues	Mass (Da)
47	23-35	1433.4
54a	108-114	779.0
54b	132-138	858.8
54c	166-171	691.0
57	155-165	1301.5
58a	100-107	977.1
58b	217-225	1037.0
60a	172-179	973.1
60b	139-148	1259.4
63	255-268	1617.9
66	36-55	2061.3
68	56-62	855.1
69	189-203	1732.9
70	226-237	1244.3
81	115-124	1237.2
88	242-254	1583.8
89	79-96	1998.2

Fig. 4. Lys-C peptide analysis/MALDI-TOF. Peptide sequences of Spot 15 Lys-C peptides as determined by Edman sequencing and accompanying MALDI-TOF MS data are listed in the figure. The data corresponds to peptides listed in the predicted amino acid sequence in Fig. 5.

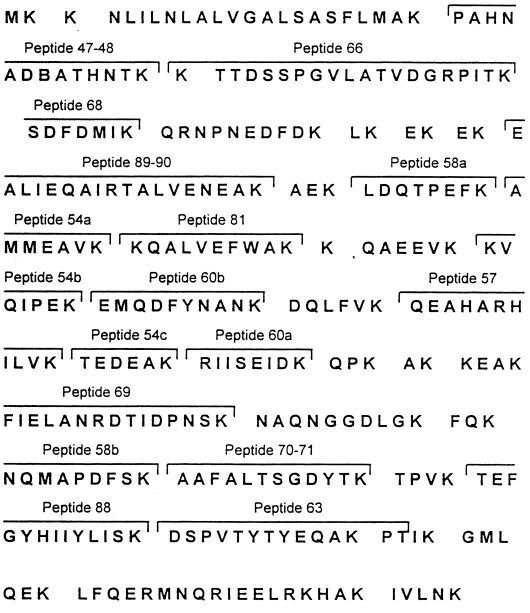

Fig. 5. Sequence of 34 000 open reading frame/Spot 15 protein. Lys-C peptides from Spot 15 are indicated in the figure, above the predicted sequence. Lower case a, b, or c following the peptide designation indicates that the peptide was obtained as a mixture of peptides. IUPAC nomenclature is used for amino acid abbreviations.

The carboxyl terminus of Spot 15 was inferred to correspond to peptide 63, because the amino acid sequence stopped at residue 14 even though the repetitive sequencing yields were more than adequate (>2 pmol/cycle) to call any subsequent residues with confidence.

The Y104B open reading frame predicted a 279 amino acid protein with a molecular mass of 34 000. The electrospray mass spectral data suggested that the Spot 15 protein resulted from a specific processing of the inferred 34 000 polypeptide product, and MALDI-TOF MS post source decay indicated that its amino terminus was generated by cleavage at amino acid residue 23 of the presumed precursor and then acetylation of the processed amino terminus. Comparison of MALDI-TOF and the ES-MS established the linear sequence of Spot 15 as residues 23–246 of the Y104B open reading frame.

The sequence of the 26 300 ES-MS species was less clear because of ambiguity in the data that could be to potential amino terminal heterogeneity and/or modification. Although the MALDI mass data could not accurately quantitate the ratio of 28 000 to 26 300, a Lys-C peptide peak was detected by MALDI which represented the sequence encompassing residues 35–55. This peptide was unusual in that the initial residues were KKT... This sequence could represent the amino terminus of the second intact protein species, 26 300, as the predicted molecular mass based upon the agreement with the mass spectrometry mass (Fig. 6), and the DNA sequence.

4. Discussion

The demonstration of *H. pylori* as a major gastroduodenal pathogen, has created the need to develop and implement new more efficient diagnostic and treatment strategies. Five general tests are used to diagnose *H. pylori* infection: three invasive tests which require endoscopy and two noninvasive tests, one measuring detection of antibodies against *H. pylori* in sera, and the other that detects gastric urease (a major *H. pylori* encoded enzyme) based on release of labelled CO_2 from labelled urea given orally. The noninvasive tests appear to be at least as

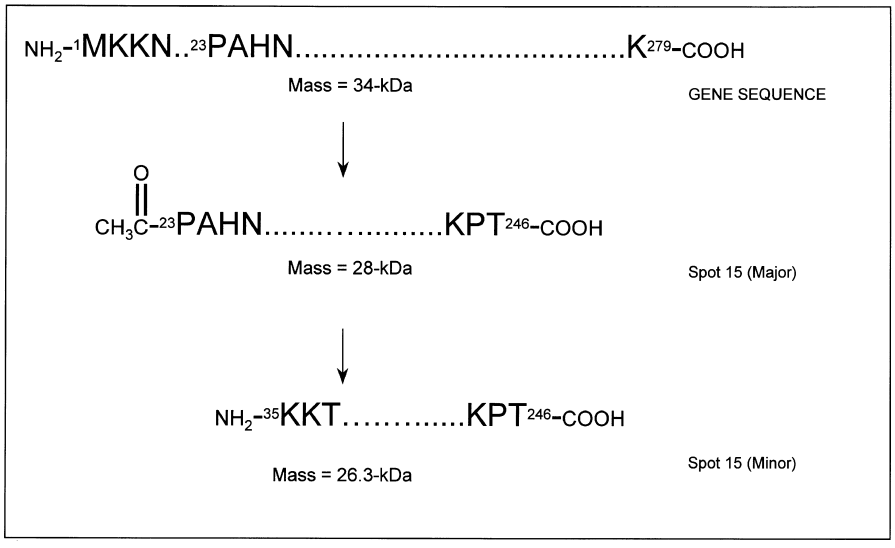

Fig. 6. Proposed processing scheme for Spot 15 protein. Predicted linear amino acid sequence for the 34 000 polypedtide is shown at the top of the figure. This sequence gives rise to the 28 000 species through processing at both the amino and carboxy termini of the polypeptide. This 28 000 species appears to be processed to a 26 300 species by further processing of the amino terminus.

accurate in predicting *H. pylori* status in untreated patients as the invasive tests (endoscopy) and to be less costly and painful to the patient [20]. While several rapid serological tests have been commercialized, none are based upon purified recombinant antigens, a strategy that offers higher sensitivity and specificity, and more robust assay design.

There is also great need for an effective anti-*H. pylori* vaccine, especially in high risk third world populations where *H. pylori* eradication by standard anti-microbial therapies is often followed by reinfection. Much attention has been focused on urease based vaccines, because of the essentiality of urease and some encouraging results with mouse-*H. felis* models, and also vacA-based vaccine in a mouse *H. pylori* model [21,22]. However, these models do not completely mimic the human condition and human clinical trials of urease have been only marginally encouraging. There is a strong sense that additional antigens may be needed for any truly effective vaccine.

Reports linking the techniques of mass spectrometry with two dimensional gel electrophoresis have given rise to the term 'proteome technologies' which refers to the systematic identification of the protein products of all genomic coding sequences [23]. Two-dimensional gel electrophoresis gives a panoramic view of a large number of these proteins. When this procedure is placed in-line with capillary LC and amino acid and DNA sequencing techniques, the ability to connect information at the protein level and genomic levels is enhanced greatly. This is particularly useful as sequence assignments based upon mass spectrometry techniques alone have not yet evolved into a straightforward process [24]. The profiling of large numbers of potentially antigenic proteins with defined serum pools raises the possibility of high throughput screening for potential diagnostic and vaccine antigens and further definition of root causes of autoimmune sequalae of some chronic infections including those caused by *H. pylori*. Amino terminal sequencing and/or proteolytic/mass spectral mapping on isolated spots represents an ideal downstream step for characterization of these potential antigens. Peptidomimetic analysis in parallel with the exploitation of recombinant DNA clone libraries could complete much of the preparation for constructing specific vaccine clones or diagnostic recombinant 'mosaic' antigens. More generally, our first analysis of numerous *H. pylori* encoded antigens [7] and the present identification of a potentially valuable antigenic protein illustrates the great power of the modern proteome approach.

Acknowledgements

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