Purification and characterization of Human Intestinal alkaline phosphatase and its role in the colonization of *Helicobacter pylori* in the duodenum


1 Department of Biotechnology, Sri Venkateswara Institute of Medical Sciences; Tirupati, India
2 Department of Pathology, Sri Venkateswara Institute of Medical Sciences; Tirupati, India
3 Department of Cardiology, Director, Sri Venkateswara Institute of Medical Sciences, Tirupati, India

* For correspondence - pvgksarma@gmail.com

**Abstract**

Intestinal alkaline phosphatase (IAP) from normal duodenum was concentrated by 0-35% ammonium sulphate and was fractionated in DEAE cellulose column and thus, partially purified IAP was purified by passing through Sephadex G-75 column in all the steps the purification was monitored through enzyme activity. The purity of the IAP was established on C-18 RPHPLC which gave single peak at a retention time of 15 minutes and SDS-PAGE analysis showed single band with molecular weight of 66 KD. Further, PAS staining confirmed the glycoprotein nature of IAP. It is very well known that IAP dephosphorylates lipid-A moiety of lipopolysaccharide layer present in the gram-negative bacteria and protects from gram negative sepsis. However, in the present study it was observed that colonisation of *H. pylori* in the duodenum showed decrease in the intestinal alkaline phosphatase activity and increased Km (IAP from normal tissue 0.98 µM of PNP/ml/min and Km =0.4µM. IAP from *H. pylori* infected tissue and Km = 0.48 µM of PNP/ml/min and Km 1 µM). The reverse-transcript PCR results indicated that, the expression of IAP was normal in both *H. pylori* infected tissue and normal intestinal tissue. Therefore, it can be concluded that colonisation of *H. pylori* in the intestine resulted in the lowering of IAP activity.

**Keywords:** *H. pylori*, Intestinal alkaline phosphatase, Sepsis, Colonisation.

**Introduction**

Alkaline phosphatase activity is found essentially in all tissues. In almost all mammals, the most abundant isozyme of alkaline phosphatase is the one found in liver, bone and kidney called non tissue specific alkaline phosphatase (AP). A second isozyme found in greatest abundance in the intestine (IAP) of all mammals in particular human beings. In humans and higher primates a third type of isozyme is present in the placenta called placental alkaline phosphatase (PLAP). Human IAP has been cloned and sequenced. The tissue non-specific AP is located on the chromosome 1 and IAP is present on chromosome 2q.37.1, (1, 2).

The levels of AP vary in ABH secretors and non-secretors. ABH non-secretors have low levels of IAP and are therefore, more prone to duodenal and peptic ulcers (3, 4). However, infections caused due to *Helicobacter pylori*, one of the most common bacterial pathogens of humans, are not dependent on ABH alleles (4). *H. pylori* colonize in the gastric mucosa, where it appears to persist throughout the host’s life unless the patient is treated. Colonization induces chronic gastric inflammation which can progress...
to variety of diseases ranging in severity from superficial gastritis and peptic ulcer to gastric cancer and mucosal associated lymphoma. Strain-specific genetic diversity has been proposed to involve in the organisms ability to cause different diseases in the infected host. *H. pylori* cause more than 90% of duodenal ulcers and up to 80% of gastric ulcers (3, 5-7). One of the major functions of IAP is protection of intestine and stomach from the infections of gram negative bacteria. One of the most life-threatening lipopolysaccharide (LPS) mediated diseases, Gram negative sepsis, is characterized by excessive production of pro-inflammatory cytokines, activation of proteolytic cascades, coagulation abnormalities (8), and hemodynamic responses, resulting in hypotension, poor tissue perfusion, and multi-organ failure (9,10). IAP dephosphorylates lipid A moiety of LPS layer of gram negative bacteria thereby, protecting the human beings from severe inflammatory responses generated due to this LPS (11). In spite of IAP clearing the infections caused due to gram negative bacteria, how *H. pylori* a gram negative bacteria overcomes the action of IAP and colonizes in the intestine, causing severe gastric and duodenal ulcers leading to cancerous condition? In order to address this question in the present study we have observed the expression of IAP in the *H. pylori* infected tissue whether the expression of IAP is down regulated or the activity of IAP is down regulated when compared with the normal IAP.

Material and Methods

Human duodenal tissue was obtained from Department of Pathology, SVIMS, and Tirupati

1. Histology: Duodenal biopsy specimens were fixed in 10% formalin. Paraffin sections were cut, Warthin Starry silver and haematoxylin and eosin stained and scored for *H. pylori* microscopically by adopting upgraded Sydney classification (12-14), were considered as the samples for the expression studies of intestinal alkaline phosphatase (IAP).

2. Reverse-Transcript Polymerase chain reaction (RT-PCR): The total mRNA from both normal duodenal tissue and *H. pylori* infected duodenal tissue and first strand synthesis was carried using AMV-reverse transcriptase (Promega). Primers for this experiment were constructed from the cDNA clone of human IAP (2), Forward primer 5’-ACTTGGG TGGATC AGGACAC-’3 and Reverse primer 5’-TCTGAGTGGCTGTGACT TGG-’3. RT-PCR was performed in the (90°C for 60s, 55°C for 30s and 72°C for 30s for 45 cycles) Eppendorf Mastercycler gradient and the obtained PCR product was analysed by running 1% agarose gel electrophoresis (15).

3. Purification and characterization of Human Intestinal Alkaline Phosphatase

3.1 Homogenization: 2gms of Human duodenal tissue was homogenized in 0.1 M Tris-HCl, pH 7.4 that contain 0.25 N Sucrose. The homogenate was centrifuged at 1200 rpm for 10 minutes at 4°C in order to remove all the cell debris. The supernatant was used as a starting material for the isolation and purification of IAP and for expression studies (16).

3.2 Enzyme Assay

The 3ml reaction mixture contains 800µl of 0.1M Carbonate-bicarbonate buffer pH 10.0, 2 ml p-Nitrophenyl phosphate and mix thoroughly. At zero time, add 200µl of enzyme. The absorbance was measured at 405nm against blank. Enzyme activity was expressed as concentration of product (PNP) formed per minute per ml. In order to check the maximum velocity of the enzyme varying concentrations of substrate p-Nitrophenyl phosphate from 1mM to 10mM were taken and enzyme assay was performed (17,19).

Determination of K_m: Michaelis-Menton constant for IAP in the normal tissue and IAP in
*Helicobacter pylori* infected duodenal tissue were determined by Hanes – Woolf plot using [S] vs. [S]/ V(17, 19).

### 3.3 Purification of Human IAP

From the crude extract the enzyme were purified in the following manner

#### 3.3.1 (NH₄)₂SO₄ Fractionation:

0-35% (NH₄)₂SO₄ was added to concentrate the IAP from the tissue extract and the solution was centrifuged at 10,000 rpm for 10 min. The pellet was suspended in 2ml of 0.1M Tris-HCl pH 7.2, and dialysed against the same buffer for overnight with intermittent changing of the buffer. The enzyme assay was performed as mentioned in 3.2. The enzyme was concentrated in speed vac concentrator.

#### 3.3.2 DEAE Cellulose Chromatography:

IAP was further fractionated on DEAE cellulose column. 35µg of 0-35% (NH₄)₂SO₄ concentrate was loaded on DEAE Cellulose column and IAP eluted with stepwise gradient of NaCl concentration prepared in 0.1M Tris-HCl pH 7.2. IAP was eluted using NaCl gradient 50mM to 200mM concentration. The peak fractions in each gradient were assayed using enzyme assay mentioned in 3.2. IAP was eluted in 100mM NaCl gradient and this was dialysed against 0.1M Tris-HCl pH 7.2. The dialysed peak fraction was concentrated using speed vac concentrator (1, 16, 23 and 26).

#### 3.3.3 Gel filtration:

25µg IAP eluted at 100mM NaCl concentration from DEAE cellulose column was loaded on Sephadex G-75 (30cm x 1.5cm) which was swollen in 0.1M Tris-HCl pH 7.2 and the IAP was eluted with 0.1M Tris-HCl pH 7.2 containing 500mM NaCl. Each peak fractions were assayed using enzyme assay mentioned in 3.2 (1, 16, 23 and 26).

#### 3.3.4 Reverse Phase HPLC purification of IAP:

5µg active fraction of IAP obtained from gel filtration was purified on reverse phase C-18 column (4.6 x 150x 5 microns) HPLC (Shimadzu) equilibrated with 0.1% trifluoroacetic acid (TFA) and elute with a linear gradient of acetonitrile containing 0.1% TFA. (1and16).

### 3.4 Characterization of IAP

**Molecular weight determination of IAP**

The molecular weight of purified IAP was determined by running 7.5% SDS-PAGE and the gel was stained with 0.125% Coomassie Brilliant blue R250. 2.5µg of purified IAP was applied, also 25µg protein from *H pylori* infected human duodenal tissue and 25µg protein from normal human duodenal tissue was applied to assess the presence of IAP isozymes. Standard molecular weight markers of molecular weights 96, 66, 43, 29, 20, and 14 KD obtained from Bangalore Genei Pvt Ltd were used in the calculation of IAP molecular weight. The glycoprotein nature of IAP was detected by staining with Periodic acid Schiff (PAS) stain (18 and 23).

The protein concentrations in all steps were determined by staining with Bradford, 1976 method (25)

### Results and Discussions

It is being observed that alkaline phosphatase present in the intestine prevents several gram negative bacteria to colonize in the intestine thus, unable to establish its pathogenicity in the host organism. However, this scenario changes dramatically in the ABH non-secretors where lower levels of IAP have been observed and are therefore, not only prone to duodenal ulcers but are also more prone to infections caused due to gram negative bacteria (4). It has been demonstrated that calf intestinal alkaline phosphatase, has provided protection against various gram-negative bacteria by dephosphorylating the lipid A moiety of LPS (20, 21). This has thrown the importance of this enzyme in clearing infections posed by gram negative bacteria (22). If high levels of IAP are expressed in the human intestine what happens to IAP activity during *H. pylori* infection? The

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The present study was aimed to identify this question. In the duodenal biopsy material *H. pylori* presence was confirmed by haematoxylin and eosin staining detected as spiral shaped organisms (13).

Expression levels of IAP in the *H. pylori* infected tissue was studied in two ways one through levels of IAP mRNA present in the normal and in the infected tissue and second by studying the IAP enzyme activity in the infected and in the normal tissue. Primers for the IAP detection were constructed from the IAP gene sequence (2).the reverse transcript polymerase chain reaction showed presence of 0.2 Kb products in both normal and infected tissue indicated the expression of IAP was normal in the infected tissue [Fig-1].

IAP was isolated and purified from normal duodenal tissue [Table-1 and Fig 2] and the purification of the enzyme was monitored through its activity. The 0-35% NH₄(SO₄)₂ concentrated IAP was purified by passing through DEAE cellulose column. IAP was eluted at 100mM NaCl concentration. IAP was further fractionated on Sephadex G-75 column and the first peak showed highly pure IAP. The purity of IAP was further confirmed on C-18 RP-HPLC where protein was eluted at a retention time of 15 minutes confirming highly hydrophilic nature of the protein. The molecular weight of the purified IAP was found to be 66 KD [Fig 3] and the glycoprotein nature was confirmed by the PAS stain (1, 2, 16, 23 and 26). Molecular mass of normal intestinal alkaline phosphatase (NIAP) is present in the serum of both secretors and non-secretors, regardless of ABO blood group. However, the high molecular mass intestinal alkaline phosphatase only appears in serum of Le (a-b+) blood group secretors and these persons are not prone to *H. pylori* infection (4). The SDS-PAGE analysis of IAP present in normal and *H. pylori* infected intestinal tissue was found to be same with no apparent change in the molecular weight of IAP this indicated that *H. pylori* infection did not affect the IAP and the same IAP was expressed in normal and in *H. pylori* infected intestinal tissue [Fig 3]. However, the enzyme activity of IAP in the *H. pylori* infected intestinal tissue was almost half of the normal IAP (IAP from normal tissue 0.98 µM of PNP/ml/min, IAP from *H. pylori* infected tissue 0.48 µM of PNP/ml/min). The Kₘ of IAP of normal tissue was found to be 0.4mM while IAP from the *H. pylori* infected intestinal tissue was found to 1mM (Table-2). This clearly indicated that the colonization of *H. pylori* in the duodenum has a profound effect on the activity of IAP. This observation can be corroborated with the fact that the patients infected with *H. pylori* when treated with Clarithromycin showed improved IAP activity (24). Therefore, it can be concluded that *H. pylori* colonisation in the duodenum resulted in the lowering of IAP activity in the intestine.
**Helicobacter pylori** in the duodenum

Fig. 2. (a) Anion exchange chromatogram (DEAE cellulose) and IAP was eluted at 100mM NaCl concentration. (b) Gel filtration on Sephadex G-75, I. IAP was eluted with 0.1M Tris-HCl pH 7.2 containing 500mM NaCl. (c) C-18 RP HPLC and IAP was eluted at a retention time of 15 minutes.

Fig. 3. Electrophoretogram showing the purification of human intestinal alkaline phosphatase 7.5% SDS-PAGE gel and the gel was stained with 0.125% coomassie brilliant blue R250. 2.5µg of purified protein was applied in 1-4 lanes, lane 5, 25µg protein from *H. pylori* infected human duodenal tissue, lane 6, 25µg protein from normal human duodenal tissue. Lane 1 IAP obtained from RP-HPLC column, lane 2 and 3 IAP obtained from gel filtration and lane 4 IAP obtained from DEAE cellulose column, lane M Molecular weight markers obtained from Bangalore Genei Pvt Ltd (96KD, 66KD, 45KD, 29KD, 21KD, 14KD).

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References


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Table 1.

<table>
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<tr>
<th>S.No.</th>
<th>Sample</th>
<th>Protein concentration µg/ml</th>
<th>Enzyme Activity micromoles of PNP/ml/min.</th>
<th>Specific activity micromoles of PNP/min/mg</th>
<th>Folds purification</th>
<th>Percent recovery</th>
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<tbody>
<tr>
<td>1</td>
<td>Crude (intestinal Homogenate)</td>
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<td>0.98</td>
<td>0.227</td>
<td>-</td>
<td></td>
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<tr>
<td>2</td>
<td>0-35% (NH₄)SO₄</td>
<td>35</td>
<td>9.10</td>
<td>0.377</td>
<td>10</td>
<td>17</td>
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<td>3</td>
<td>DEAE cellulose purified fraction (IAP)</td>
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<td>18.40</td>
<td>4.03</td>
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<td>Sephadex G-75 (30cm x1.5cm)</td>
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<td>27.60</td>
<td>4.87</td>
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<td>12</td>
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<tr>
<td>5</td>
<td>C-18 RP-HPLC (4.6 x 150 x 5 microns)</td>
<td>4.0</td>
<td>45</td>
<td>5.27</td>
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Table 2.

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<th>Tissue</th>
<th>Activity µM of PNP/ml/min</th>
<th>Kₘ (mM of PNPP)</th>
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</thead>
<tbody>
<tr>
<td>Normal duodenal tissue</td>
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<td>0.4</td>
</tr>
<tr>
<td>H. pylori infected duodenal tissue</td>
<td>0.48</td>
<td>1</td>
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</table>

Table 2.


