The Laryngoscope
Lippincott Williams & Wilkins

Activity/Stability of Human Pepsin: Implications for Reflux Attributed Laryngeal Disease

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Objectives/Hypothesis: Exposure of laryngeal epithelia to pepsin during extra-esophageal reflux causes depletion of laryngeal protective proteins, carbonic anhydrase isoenzyme III (CAIII), and squamous epithelial stress protein Sep70. The first objective of this study was to determine whether pepsin has to be enzymatically active to deplete these proteins. The second objective was to investigate the effect of pH on the activity and stability of human pepsin 3b under conditions that might be found in the human esophagus and larynx.

Study Design: Prospective translational research study.

Methods: An established porcine in vitro model was used to examine the effect of active/inactive pepsin on laryngeal CAIII and Sep70 protein levels. The activity and stability of pepsin was determined by kinetic assay, measuring the rate of hydrolysis of a synthetic pepsin-specific substrate after incubation at various pH values for increasing duration.

Results: Active pepsin is required to deplete laryngeal CAIII and Sep70. Pepsin has maximum activity at pH 2.0 and is inactive at pH 6.5 or higher. Although pepsin is inactive at pH 6.5 and above, it remains stable until pH 8.0 and can be reactivated when the pH is reduced. Pepsin is stable for at least 24 hours at pH 7.0, 37°C and retains 79% ± 11% of its original activity after re-acidification at pH 3.0.

Conclusions: Detectable levels of pepsin remain in laryngeal epithelia after a reflux event. Pepsin bound there would be enzymatically inactive because the mean pH of the laryngopharynx is pH 6.8. Significantly, pepsin could remain in a form that would be reactivated by a subsequent decrease in pH, such as would occur during an acidic reflux event or possibly after uptake into intracellular compartments of lower pH.

Key Words: Activity, carbonic anhydrase isoenzyme III, laryngopharyngeal reflux, pepsin, pH profile, squamous epithelial stress protein 70, stability.

***Laryngoscope,* 117:1036–1039, 2007**

INTRODUCTION

Laryngopharyngeal reflux (LPR), defined as the backflow of gastric contents into the laryngopharynx, contributes to several otolaryngologic inflammatory disorders and neoplastic diseases.[1,2] It is has been reported in up to 10% of patients referred to otolaryngologists for treatment and in 50% of patients with laryngeal and voice disorders.[1] Damage to the laryngeal epithelium is believed central in the development of reflux-attributed laryngeal disease, thought to occur after a breakdown in defense. Despite recent advances in diagnosing LPR, very little is known regarding its effect on the biology and biochemistry of the laryngeal epithelium.

For several years, a presumed relationship between LPR and laryngeal lesions and disease contributed to a focus on reflux pH as the principal diagnostic and therapeutic target for LPR. Double-probe pH monitoring is commonly used to diagnose acid reflux into the laryngopharynx; however, this does not address the role of non/weak acid reflux, which is now known to occur, causing symptoms and mucosal injury.[3–6] Proton pump inhibitors (PPIs), the most potent form of acid suppression therapy available, are widely prescribed to treat LPR, yet even high doses can often be inadequate to treat many patients with LPR-attributed symptoms and disease.[3,7] This is because PPI therapy decreases the hydrogen ion concentration

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Editor's Note: This Manuscript was accepted for publication January 30, 2007.

Presented at the Triological Society Combined Sections Meeting, Marco Island, Florida, U.S.A, February 14–18, 2007.

This research study was sponsored in part by the Department of Otolaryngology and Communication Sciences, Medical College of Wisconsin, Milwaukee, Wisconsin, U.S.A.; and the Department of Otolaryngology, Wake Forest University Baptist Medical Center, Winston-Salem, North Carolina, U.S.A.

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DOI: 10.1097/MLG.0b013e31804154c3

of the refluxed fluid but not the number or duration of reflux events.[3] With new evidence that symptoms and injury can be caused by non/weak acid reflux (using combined multichannel intraluminal impedance pH), it is clear that PPI therapy alone has limited ability to protect patients from LPR-induced damage.

It is likely that patients with incomplete response to acid suppression may have significant involvement from other components of the gastric refluxate, such as pepsin and bile acids, both of which can injure the laryngeal/esophageal epithelium at non/weakly acidic pH. Pepsin is the major proteolytic enzyme produced in the stomach by gastric chief cells. We have demonstrated that pepsin is present in the laryngeal epithelium of patients with reflux-attributed laryngeal disease but is absent in normal control subjects.[8] Furthermore, we have determined a significant association between the presence of pepsin in laryngeal epithelia of LPR patients and the depletion of two laryngeal protective proteins, carbonic anhydrase isoenzyme III (CAIII) and squamous epithelial stress protein Sep70.[8–10] Using an established porcine in vitro model, we have demonstrated that exposure of laryngeal mucosa to pepsin, although not to low pH alone, causes depletion of CAIII and Sep70 protein levels. These findings suggest that the pepsin present in laryngeal epithelia of LPR patients is likely to be the causal factor for the observed depletion of CAIII and Sep70 proteins.

The objectives of this study were to 1) determine whether enzymatically active pepsin is required to deplete laryngeal CAIII and Sep70 protective proteins and 2) establish whether pepsin, present in the laryngeal epithelium of LPR patients (which is presumably inactive because the mean pH of the laryngopharynx is pH 6.8, and pepsin is maximally active at pH 2.0 and inactive at pH $\geq$6.5), is stable and thus could be reactivated after a decrease in pH, as would occur by a subsequent acidic reflux event. To accomplish these objectives, the affect of exposure of laryngeal mucosa to active/inactive pepsin (porcine pepsin A, 1 mg/mL, $\pm$ pepstatin, 23 μg/mL) on CAIII and Sep70 protein levels was investigated using the established porcine in vitro model.[9] The effect of pH on activity and stability of human pepsin 3b was measured by kinetic assay by measuring the rate of hydrolysis of a specific synthetic substrate for human pepsin 3b. Hydrolysis of the peptide substrate was measured at pH 1.5 to pH 8.0 to determine peptic activity at each pH. The stability of pepsin was measured by incubating the enzyme at pH 2.0 to pH 8.0 for 24 hours at 37°C, after which the rate of hydrolysis of the substrate was measured at pH 3.0.

METHODS

Isolation of Pepsin 3b

Human gastric juice was collected from patients attending the laryngology outpatient clinic at Froedtert Hospital, Milwaukee, WI, scheduled to have a transnasal esophagoscopy for clinical indications. Approximately 5 to 10 mL of gastric juice was aspirated from patients at the end of their scheduled procedure. Gastric juice samples were pooled and pepsin 3b isolated by anion exchange chromatography. This study was approved by the Institutional Review Board at the Medical College of Wisconsin, study number PRO 00004759, and informed consent was obtained from all participants.

Gastric juice was initially filtered through a piece of 113 V, 320-mm diameter Whatman filter paper (Fisher Scientific, Pittsburgh, PA) to remove mucoid and particulate matter. The filtrate was then dialyzed against 50 mmol/L sodium acetate buffer, pH 4.1 at 4°C. Partial purification of pepsins was performed using a diethyl amino ethyl cellulose column (DE52, 2.5 $\times$ 10 cm, Whatman, Inc., NJ) equilibrated with dialysis buffer at 4°C. Negatively charged proteins, including pepsin 3b, were eluted from the DE52 column with salt (sodium acetate/1 mol/L NaCl at 2 mL/min). Fractions containing protein (determined by measuring optical density at 280 nm) were pooled and dialyzed against 50 mmol/L sodium acetate at 4°C to remove all salt before performing the second high-resolution anion exchange chromatography with a Pharmacia Mono Q, 0.5 $\times$ 5 cm, column (GE Health Care, Piscataway, NJ) developed with a linear gradient of 0.09 to 1.0 mol/L NaCl in 50 mmol/L sodium acetate, pH 4.1, over 30 minutes at 0.75 mL/minute. Pepsin 3b was identified by chromatography pattern, isolated, and stored at -20°C in 50% glycerol.[11]

Synthetic Peptide Substrate for Pepsin

The synthetic peptide substrate for pepsin (Lys-Pro-Ala-Glu-Phe-PNP-Arg-Leu-COOH, molecular weight = 1,052.18; PNP = paranitrophenylalanine) was synthesized in the Protein Analysis Core Laboratory at Wake Forest University Health Sciences, Winston-Salem, NC. This peptide is cleaved specifically by pepsin between Phe-5 and PNP-6. Cleavage was monitored by decreasing absorbance at 300 nm.[12]

Porcine In Vitro Model

Porcine laryngeal mucosa was exposed to pepsin (porcine pepsin A, 1 mg/mL) $\pm$ pepstatin (23 μg/mL, an irreversible inhibitor of pepsin) using an established in vitro organ culture model.[9] The activity of pepsin $\pm$ pepstatin was measured by kinetic assay (rate of hydrolysis of the synthetic peptide substrate)[12] before it was applied to the porcine laryngeal mucosa.

Kinetic Assays

The rate of hydrolysis of the synthetic peptide substrate (70 μmol/L) by pepsin (0.1 μg/mL) at pH 1.5 to 8.0 was measured by kinetic assay at 300 nm. Pepsin was prepared in sodium acetate, 0.2 mol/L, pH 4.1. HCl was added to obtain each pH, taking note of the amount added and of what concentration so that the final [Cl^-] could be balanced using 1 mol/L NaCl. The rate of the first 10% hydrolysis was calculated for each reaction and a graph of the mean ($\pm$SEM) rate (nmol/L per min, n = 2) for each pH plotted.

To investigate the stability of human pepsin 3b, pepsin was prepared in sodium acetate buffer (0.2 mol/L) with the pH adjusted to pH 2.0 to 8.0 and [Cl^-] balanced with NaCl. After incubation at 37°C for 24 hours, the rate of hydrolysis of the peptide substrate (70 μmol/L, prepared in sodium formate, 0.1 mol/L, pH 3.0) was measured by the decrease in absorbance at 300 nm at pH 3.0. The mean ($\pm$SEM) rate (nmol/L per min, n = 3) after incubation at 37°C for 24 hours at each pH was compared with the mean ($\pm$SEM) rate (nmol/L per min, n = 2) at each pH without incubation for 24 hours at 37°C.

The pH was measured before and after the 24-hour incubation at 37°C. It did not change.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Porcine laryngeal specimens were homogenized and lysed as described by Axford et al.[13] Ten micrograms of total protein was separated on either a 10% or 12% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis, transferred to a nitrocellulose

membrane (Amersham International, Little Chalfont, UK), and probed with either a mouse monoclonal antihuman CAIII diluted 1:1,500 (Nanogen, Toronto, Canada) or a mouse monoclonal to antihuman Sep70 diluted 1:8,000 (Moravian Biotechnology, Bruno, Czech Republic). All Western blots were stripped using a Re-Blot Plus strong antibody stripping solution (Chemicon International, Temecula, CA), and nonspecific sites were blocked using nonfat milk and reprobed for actin using the monoclonal anti-actin diluted 1:10,000 (Oncogene Research Products, San Diego, CA). This was performed to ensure equal loading of total protein in all lanes. All antibodies were diluted in phosphate-buffered saline containing 1% (vol/vol) Tween-20. Detection was by enhanced chemiluminescence. The specificity and sensitivity of the antihuman CAIII and Sep70 antibodies have previously been described.[13,14]

RESULTS

Western blot analysis revealed that exposure of laryngeal mucosa to active, but not inactive, pepsin results in the depletion of laryngeal CAIII and Sep70 protein levels (Fig. 1). Human pepsin 3b has maximum activity at pH 2.0 and remains active until pH 6.5 (Fig. 2). Although pepsin is inactive at pH 6.5 and above, it remains stable and can be reactivated after a decrease in pH (Fig. 3). We have demonstrated that human pepsin 3b can be incubated at pH 7.0 for up to 24 hours at 37°C and then be reactivated when the pH is reduced to 3.0 to obtain 79 ± 11% of its original activity. Pepsin retains 70% to 80% of its original activity whether it is incubated at pH 7.0 for 5 minutes or 24 hours at 37°C. Pepsin is not irreversibly inactivated until pH 8.0.

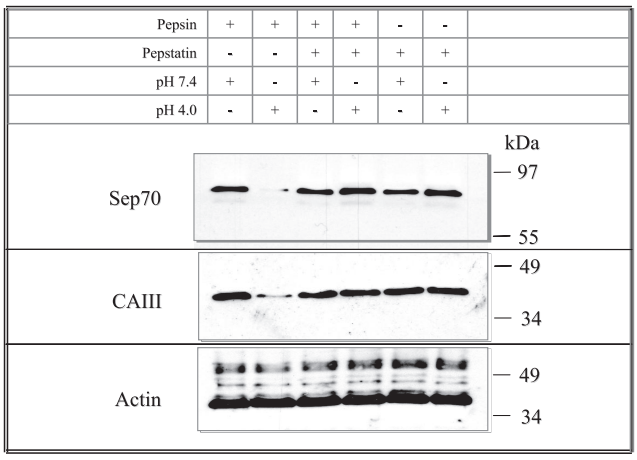

Fig. 1. Effect of active/inactive pepsin on laryngeal Sep70 and CAIII protein levels. We have previously shown that laryngeal Sep70 protein levels are depleted after exposure to pepsin (porcine pepsin A, 1 mg/mL) at pH 4.0 for 3 hours.[10] When pepsin is inactivated with pepstatin (23 μg/mL), this reduction in Sep70 levels is not observed. We have also shown a decrease in laryngeal CAIII protein levels after exposure to pepsin at pH 4.0 for 20 minutes (followed by 4 hr incubation in media at pH 7.4).[9] In presence of pepstatin, CAIII is not depleted. Thus, active, and not inactive, pepsin causes depletion in laryngeal Sep70 and CAIII protein levels. Blots were stripped and reprobed for actin to show equal loading of total protein. CAIIIrp = carbonic anhydrase isoenzyme III recombinant protein (positive control); LB = loading buffer.

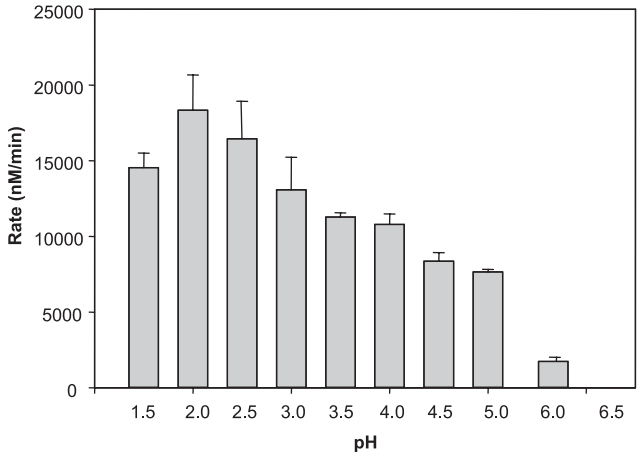

Fig. 2. Effect of pH on activity of human pepsin 3b. Rate of hydrolysis of pepsin-specific peptide substrate (70 μmol/L) by human pepsin 3b (0.1 μg/mL) at pH 1.5 to 8.0 was measured by kinetic assay at 300 nm. Pepsin has maximum activity at pH 2.0 and is inactive at pH 6.5 (n = 2).

DISCUSSION

We have demonstrated that exposure of laryngeal mucosa to active, but not inactive, pepsin results in the depletion of laryngeal CAIII and Sep70 protective proteins. Thus, patients with reflux-attributed laryngeal disease, who have depleted levels of these proteins, must have been exposed to active pepsin.

In 1965, Piper and Fenton[15] demonstrated peptic activity in gastric juice using proteolysis of radioiodinated serum albumin at pH 2.0 as a measure of pepsin activity. They reported maximal activity at pH 2.0 and demonstrated that pepsin was inactive at pH 6.5. The authors also reported the stability of pepsin by incubating gastric juice at 37°C for 10 minutes at varying pH levels before titrating to pH 2.0. Of interest, although pepsin is inactive

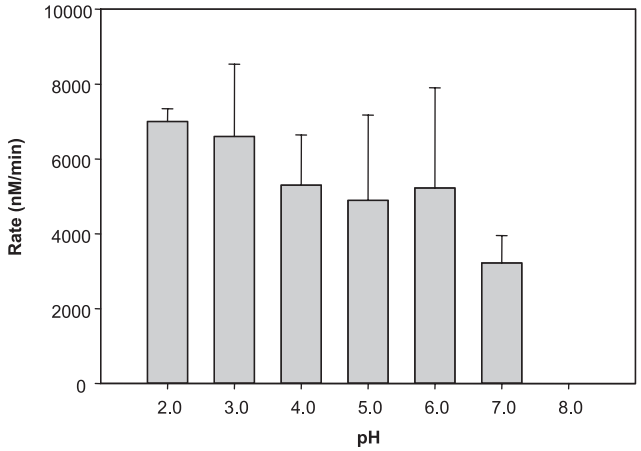

Fig. 3. Stability of human pepsin 3b. Pepsin was incubated at indicated pH (2.0–8.0) for 24 hours at 37°C, after which rate of hydrolysis of peptide substrate (70 μmol/L) was measured at pH 3.0. Although pepsin (0.1 μg/mL) is inactive at neutral pH, it is stable. After incubation at pH 7.0 for 24 hours, pepsin can be reactivated by decreasing the pH to 3.0, retaining 79% ± 11% of its original activity (n = 3).

at pH 6.5, it was shown to be stable at this pH because it could be reactivated when the pH was dropped to pH 2.0, retaining 70% of its original activity.

We repeated this study using pepsin 3b isolated from human gastric juice and a pepsin-specific peptide substrate. In addition, we investigated whether pepsin would remain stable for a longer time period. Piper and Fenton incubated pepsin at various pHs for 10 minutes at 37°C, after which they decreased the pH to 2.0 and measured peptic activity. In this study, we incubated pepsin at pH 2.0 to 8.0 for 24 hours at 37°C and then decreased the pH to 3.0 and measured peptic activity. Our data are consistent with that of Piper and Fenton and further reveal that pepsin remains stable at pH 7.0 for at least 24 hours at 37°C, retaining 79% ± 11% of its original activity on re-acidification. Thus, as long as pepsin remains below pH 8.0, it remains stable and thus has the potential to be reactivated if the pH significantly decreases again.

This is of clinical importance because pepsin, which can be detected in laryngeal epithelia after a reflux event,[8,10] could be inactive because the mean pH of the laryngopharynx is 6.8. However, even if pepsin present in the laryngeal tissue is enzymatically inactive, it would potentially remain stable and thus could be reactivated after a subsequent acidic reflux event or once taken up by laryngeal epithelial cells into acidic intracellular compartments. Recent electron microscopy studies demonstrating colocalization of pepsin with specific markers for the receptor mediated endocytic pathway (transferrin and clathrin) have confirmed our initial confocal microscopy findings that pepsin is taken up by laryngeal epithelial cells by a specific process that appears to be receptor mediated (unpublished data).[10] Thus, pepsin would be expected to be found in intracellular compartments such as the transreticular Golgi or lysosomes, which are approximately pH 5 and 4, respectively. Ongoing studies in the lead author's research laboratory will further delineate the uptake of pepsin by laryngeal epithelial cells and its intracellular path.

CONCLUSION

Pepsin can be detected in laryngeal epithelia after a reflux event. The results of this study show that pepsin that remains in the laryngopharynx after a reflux event is reversibly inactivated because the mean pH of the laryngopharynx is 6.8, a pH at which the enzymatic activity of pepsin is undetectable. Significantly, these data show that pepsin may remain in a state that can be reversibly reactivated until the pH of the environment increases above pH 8.0. Thus, pepsin could remain stable in the larynx and be reactivated at a later time after a decrease in pH, such as could be caused by a subsequent acidic reflux event. Pepsin could also potentially be reactivated after uptake by the laryngeal epithelial cells into intracellular compartments of lower pH such as the transreticular Golgi network or lysosomes.

BIBLIOGRAPHY

1. Koufman JA. The otolaryngologic manifestations of gastroesophageal reflux disease (GERD): a clinical investigation of 225 patients using ambulatory 24-hour pH monitoring and an experimental investigation of the role of acid and pepsin in the development of laryngeal injury. *Laryngoscope* 1991;101(Suppl 53):1–78.
2. Qadeer MA, Colabianchi N, Vaezi MF. Is GERD a risk factor for laryngeal cancer? *Laryngoscope* 2005;115:486–491.
3. Tamhankar AP, Peters JH, Portale G, et al. Omeprazole does not reduce gastroesophageal reflux: new insights using multichannel intraluminal impedance technology. *J Gastrointest Surg* 2004;8:890–898.
4. Kawamura O, Aslam M, Rittmann T, et al. Physical and pH properties of gastroesophagopharyngeal refluxate: a 24-hour simultaneous ambulatory impedance and pH monitoring study. *Am J Gastroenterol* 2004;99:1000–1010.
5. Oelschlager BK, Quiroga E, Isch JA, et al. Gastroesophageal and pharyngeal reflux detection using impedance and 24-hour pH monitoring in asymptomatic subjects: defining the normal environment. *J Gastrointest Surg* 2006;10:54–62.
6. Mainie I, Tutuian R, Shay S, et al. Acid and non-acid reflux in patients with persistent symptoms despite acid suppressive therapy. A multicentre study using combined ambulatory impedance-pH monitoring. *Gut* 2006;55:1398–1402.
7. Castell DO, Kahrilas PJ, Richter JE, et al. Esomeprazole (40 mg) compared with lansoprazole (30 mg) in the treatment of erosive esophagitis. *Am J Gastroenterol* 2002;97:575–583.
8. Johnston N, Knight J, Dettmar PW, et al. Pepsin and carbonic anhydrase isoenzyme III as diagnostic markers for laryngopharyngeal reflux disease. *Laryngoscope* 2004;114:2129–2134.
9. Johnston N, Bulmer D, Gill GA, et al. Cell biology of laryngeal epithelial defenses in health and disease: further studies. *Ann Otol Rhinol Laryngol* 2003;112:481–491.
10. Johnston N, Dettmar PW, Lively MO, et al. Effect of pepsin on laryngeal stress protein (Sep70, Sep53, and Hsp70) response: role in laryngopharyngeal reflux disease. *Ann Otol Rhinol Laryngol* 2006;115:47–58.
11. Jones AT, Balan KK, Jenkins SA, et al. Assay of gastricsin and individual pepsins in human gastric juice. *J Clin Pathol* 1993;46:254–258.
12. Dunn BM, Jimenez M, Parten BF, et al. A systematic series of synthetic chromophoric substrates for aspartic proteinases. *Biochem J* 1986;237:899–906.
13. Axford SE, Sharp N, Ross PE, et al. Cell biology of laryngeal epithelial defenses in health and disease: preliminary studies. *Ann Otol Rhinol Laryngol* 2001;110:1099–1108.
14. Yagui-Beltran A, Craig AL, Lawrie L, et al. The human oesophageal squamous epithelium exhibits a novel type of heat shock protein response. *Eur J Biochem* 2001;268:5343–5355.
15. Piper DW, Fenton BH. pH stability and activity curves of pepsin with special reference to their clinical importance. *Gut* 1965;6:506–508.