Journal of Undergraduate Chemistry Research, **2024**, 23(1), 1

EVALUATION OF THE ISOELECTRIC POINTS OF PLANT AND PATHOGENIC PECTIN DEGRADING ENZYMES

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Abstract

Highly esterified polygalacturonan (PGA) makes up a significant portion of plant cell wall pectin and is the primary barrier against fungal and bacterial phytopathogens. However, to allow for normal cell growth, the action of pectin methylesterases (PMEs) is thought to be necessary prior to the hydrolysis action of polygalacturonases (PGs). The action of PMEs is also believed to lower the pH of the cell wall environment, but little has been explored about the possible role the change in pH may play. Using calculated isoelectric points as an estimation for the pH of optimal activity, the most comprehensive analysis of PME and PG sequences was performed, including 198 protein sequences from the most significant crops and crop disease pathogens, to better understand the cooperative action of PMEs and PGs. The results suggest that PME activity may lower the cell wall pH to optimize PG activity for both plant and pathogen.

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Keywords: Pectin, polygalacturonase, pectin methylesterase, crop disease, phytopathogens
Received: December 9, 2023 Accepted: January 16, 2024 Published: January 22, 2024

Introduction.

With a population of nearly 8 billion and annual growth of 1%, the need for worldwide agricultural productivity has never been greater than it is today.[1] Despite the increasing demands, greater than $200 billion dollars is lost annually to crop disease.[2] Whether in the fields, storage, or transit, bacteria and fungi are constant threats to plants and produce. The plant cell wall, a pectin network comprised of variable polysaccharides, is the first and primary defense against phytopathogens.[3] Interestingly, however, this rigid cellular structure is an obstacle for normal plant growth and fruit ripening. Consequently, the plant cell wall serves as a fascinatingly complex battle ground between plant and pathogen, each with multiple enzymes that compete to either protect, expose, or loosen the cell wall.

The pectin network is principally composed of polygalacturonan (polygalacturonic acid or PGA). PGA is vulnerable to the action of endogenous or pathogenic polygalacturonases (PGs) which cleave α-1,4 glycosidic bonds. Endogenous PG activity results in the softening of the cell wall and is necessary for cell growth and fruit ripening. Pathogenic PG activity more extensively creates holes in the pectin network, providing a means for the release of more enzymes into the host cell to provide nutrients for the pathogen.[4-7] Although plants do possess polygalacturonase inhibitor proteins (PGIPs) against some pathogenic PGs, the specificity is not well understood and no PGIP seems to be universally effective.[8-11] Additionally, PGA is often methyl esterified. The esterification seems to protect the PGA from both endogenous and pathogenic PG activity. However, both plants and pathogens have pectin methylesterases (PMEs) that de-esterify segments of PGA to allow for PG activity.[12-14]

The reality that both plant and pathogens possess similar functioning PMEs and PGs, the endogenous ones used to facilitate normal growth cycles and pathogenic ones used to irreversibly weaken the cell wall, emphasizes the need for a more comprehensive understanding of how these enzyme systems interact. This present study attempts to contribute to our understanding of the role of plant cell wall pH. Several pathogens, like *Aspergillus* fungi, have multiple PGs,[10] and studies have been performed to evaluate the pH of their optimal activities.[10,15-26] The results of these studies suggest that if a pathogen has several PGs, they are likely to be optimally active at differing pHs. This has been suggested to be an evolutionary advancement enabling the pathogen to be more versatile. These studies, although quite valuable, are few in number presumably due to the challenge of protein purification. So, we desire a more comprehensive evaluation of optimal pH activity among important crops and crop disease pathogens.

Additionally, there has been only minimal mention in the literature of the effect of PME activity in lowering the pH of the plant cell wall environment.[27] The de-esterification process exposes carboxylic acids which, in turn, lowers the ambient pH. Therefore, it is possible that possessing a set of PGs optimally active at different pH's, may not only provide a pathogen versatility against various plant hosts, but also allow it to stay viable indefinitely throughout the degradation process.

Generally, PGs with acidic isoelectric points (pI) are most active at acidic pHs, while those with alkaline pIs are optimally active under alkaline conditions. The isoelectric focusing-measured pI of *Aspergillus aculeatus* PG is in the range of 4.2-4.6, and the enzyme has been determined to be optimally active at pH 5.[28] PMEs are also optimally active within the range of their isoelectric points and have been shown to exhibit a pH-dependent mechanism of action.[29] Interestingly, studies of *Aspergillus* pectin lyase crystal structure have shown that active-site aspartate residues critical for its activity are involved in a pH-triggered conformational change.[30] However, despite having pH-responsive active site aspartate residues that participate in an acid-base mechanism, PGs do not exhibit pH-dependent structural changes, suggesting a relatively rigid conformation. Notably, PGs crystallized at different pH levels show differences in the T1 loop, which contains a tyrosine residue crucial for catalysis. The tyrosine residue has been suggested to play a role in transition state stabilization.[28] All these

studies highlight the crucial role of pH in the structure and activity of PGs and PMEs, and that calculating isoelectric points could be a helpful tool for estimating their optimal pH and mechanism of action for large numbers of enzymes. Therefore, this current work provides the first comprehensive, large-scale evaluation and comparison of isoelectric points among plant and phytopathogenic PGs and PMEs, in the hope that the patterns observed may provide insight into both the normal function of the plant cell wall metabolism and pathogenesis.

Methods.

A thorough sampling of plant and pathogenic PGs and PMEs was selected from the National Center for Biotechnology Information protein database.[31] To guide the selection process of the pathogens, the list attempted to include the most significant bacterial and fungal crop pathogens,[32,33] and those for which pH optimum studies had been performed.[10,15-26] The investigation identified 58 PGs and 60 PMEs with mature sequences characterized in the database. The plants were chosen to include the most financially important crops as gleaned from the United Nations Food and Agriculture Organization's database.[34] The investigation identified 33 PGs and 47 PMEs with mature sequences characterized in the database. The isoelectric points for the mature sequence of each protein were estimated using the publicly available Protein Calculator 3.4v.[35] Descriptive statistics and appropriate F-tests and t-tests were performed in Microsoft Excel.

Results and Discussion.

Experimental pH optimum data exists for 13 pathogenic PGs for which a characterized mature sequence is known. The theoretically determined pIs were compared with their experimentally observed pH optima using a paired t-test. Their statistical similarity was at the edge of significance (p-value ≈ 0.05) with mean values of 5.3 for the pIs and 4.8 for the pH optima. Considering that most of the experimental reports evaluated activity in 0.5 pH unit increments and some at only 1.0 pH unit increments, a mean difference of 0.5 is encouraging for the validity of this study. Additionally, the theoretically computed pIs of the PGs from *A. aculeatus* agree to within 0.5 pH units of their experimentally estimated pIs determined through isoelectric focusing reported in the literature,[28] supporting the use of theoretically calculated pIs as reasonable estimations for the purpose of this study.

Mean pIs and standard deviations were calculated for each group of proteins (Table 1). Plant PMEs were consistently alkaline, with a mean pI of just under 9 (8.7 +/- 0.8). When comparing these with the pathogenic PMEs, it became immediately apparent that the pathogenic PMEs existed in two significantly different pI classes, referred to as alkaline (mean pI = 8.7 +/- 0.4) and acidic (mean pI = 5.3 +/- 0.7). Interestingly, the pIs of the alkaline pathogenic PMEs were similar to the plant PMEs (p-value = 0.60). The acidic pathogenic PMEs, on the other hand, were dramatically different from either the plant PMEs or the alkaline pathogenic PMEs, (p-value = $1x10^{-32}$ and $1x10^{-29}$, respectively).

Table 1: The mean and standard deviations of each group of PME and PGs are shown.

	PMEs			PGs	
	Plant	Acidic Pathogenic	Akaline Pathogenic	Plant	Pathogenic
Number of Proteins	47	32	28	33	58
Mean pI	8.7	5.3	8.7	7.7	5.9
Standard Deviation	0.8	0.7	0.4	1.0	1.3

The mean pIs of the plant PGs (7.7 +/-1.0) and pathogenic PGs (5.9 +/- 1.3) were also statistically different from each other (p-value = $4x10^{-10}$) with the pathogenic PGs being significantly more acidic. However different, both PG groups demonstrated a much larger range of pI values than what was seen among the PMEs (Figure 1), further suggesting a different mechanism of pH dependence between these two classes of enzymes.

Considering normal plant metabolism, it is evident that the ubiquitous alkaline pH of highly esterified polygalacturonan is suitable for the action of the plant's endogenous PMEs. The action of the plant PMEs both frees up portions of deesterified PGA for hydrolysis by PGs and lowers the ambient pH to what is likely more suitable for the endogenous PGs. Additionally, as the pH drops, it is reasonable to expect that the plant PMEs lose activity. In this manner, a consequential decrease in pH may provide a sort of self-regulation, preventing uncontrolled deesterification by endogenous PMEs, which would place the cell wall structure in a state of significant vulnerability.

Regarding pathogenesis, it is interesting to note the two very different groups of PMEs, acidic and alkaline. Seeing that the pathogenic alkaline PMEs have statistically similar pIs to plant PMEs, it seems reasonable to conclude that the alkaline PMEs provide some phytopathogens with the capability of deesterifying otherwise healthy pectin. Furthermore, the pathogenic PGs are significantly more acidic than plant PGs, which may suggest the need for more acidic PMEs that are capable of continuing the gross deesterification and to further drive down the pH for the optimal functioning of the pathogenic PGs.

Consequently, the statistical similarity of the plant and pathogenic alkaline PMEs and discreetly different pIs of the other groups of enzymes suggests a pattern of action for the endogenous plant enzymes as well as for the pathogenic enzymes. Healthy esterified PGA is processed by endogenous PMEs which properly prepares the PGA for the work of plant PGs by both lowering the pH slightly and deesterifying the portions of the cell wall.

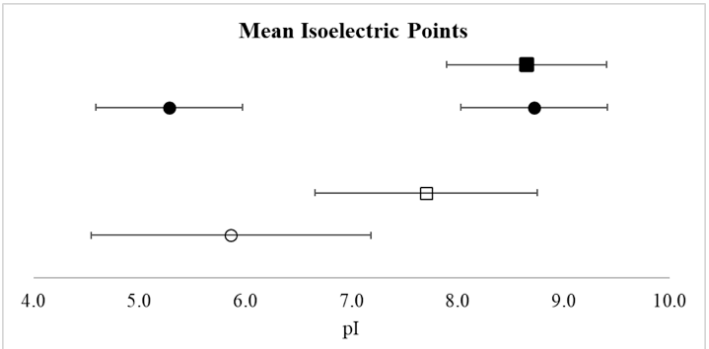

Figure 1: The mean isoelectric point for plant PMEs (solid square), acidic and alkaline pathogenic PMEs (solid circle), plant PGs (open square), and pathogenic PGs (open circle) are shown with standard deviations indicated with error bars

Phytopathogens, on the other hand, may possess alkaline PMEs to begin deesterification and acidic PMEs to continue preparing the PGA for more comprehensive hydrolysis by the acid pathogenic PGs leading to the ultimate demise of the plant.

Future work will include polygalacturonase inhibitor proteins (PGIPs) which plants express to slow the action of pathogenic PGs. By evaluating the pIs of plant PGIPs, it may be possible to better envision at what point during pathogenesis they would begin to intervene. There are also a variety of plant derived PME inhibitors that may be considered, however, their structures and sequences may be too diverse and inconsistent for this methodology to be useful.

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