The Enduring Controversies Concerning the Process of Protein Digestion in *Nepenthes* (Nepenthaceae)

Christopher K. Frazier • Department of Biology • University of New Mexico • Albuquerque, NM 87131 USA • cfrazie@unm.edu

Keywords: carnivory: *Nepenthes* — chemistry: *Nepenthes* — history.

Introduction

Plants typically make their protein using carbon incorporated from photosynthesis and nitrogen taken up from inorganic mineral in the soil. In contrast, animals, fungi and carnivorous plants make their protein—at least in part—from materials derived from the tissues and enzymes of other organisms. In these organisms, protein sources are broken down by enzymes, called proteases, into their basic building blocks, called amino acids, which are then absorbed. There is no doubt that tropical pitcher plants (*Nepenthes*) eat prey: they attract it, kill it and absorb the products of digestion. How *Nepenthes* accomplishes this process has been the object of study for 125 years. An enduring controversy with respect to *Nepenthes* digestion concerns whether the digestion is effected by proteases produced by the plant itself (endogenous) or from the activities of bacteria or other free-living sources in the pitcher fluid. In this review, I trace the history of this study, pointing to some of the major accomplishments in our increased understanding of the process and some of the important questions yet to be resolved.

The Early Years: 1874-1942

Inspired directly by Charles Darwin, J. D. Hooker (1874) was the first to document that *Nepenthes* is carnivorous. He established the digestive activity of the fluid, stating that egg-white, meat and cartilage all showed unmistakable evidence of disintegration within 24 hours. Others confirmed Hooker's observations on digestion and added the important observation that acidification increased the digestive activity (Gorup-Bensanez, 1874; Tait, 1875; Gorup-Bensanez & Will, 1876). Vines (1877) showed that extracts from the pitchers themselves could digest fibrin, but only when acidified. Pre-incubation of the pitcher with dilute acid increased the digestive activity. Evidence mounted that *Nepenthes* produces the digestive enzymes endogenously and that the digestive process is remarkably like that of our own stomach.

This theory was disputed first by Dubois (1890) in France and then by Tischutkin (1892) in Russia. Their two studies were similar in that they both found that the fluid of opened pitchers effected digestion, but that of unopened pitchers did not. While this was not a new observation (Tait had already noticed this in 1875), their conclusion was novel. They believed digestion was not accomplished by the secretion of the pitcher, but rather by the action of bacteria and infusia that gather in the opened pitchers. Goebel (1893) and Vines (1897) countered the bacterial hypothesis with additional evidence. Goebel showed that opened pitchers effected digestion even when bacteria were not present and that the fluid from unopened pitchers, when acidified, digested protein. Vines (1897) showed that fluid from unopened pitchers digested protein when 0.2% HCl was added. Acidified water, on the other hand, did not digest protein. Further experiments by Vines demonstrated that digestion occured even when bacterial poisons were present. Couvreur (1900)

claimed that Vines' results were due to the interaction of reagents, but he offered no rigorous support for his theory, nor were his arguments sound. In 1901, Vines suggested the name nepenthin for the *Nepenthes* digestive enzyme.

The relative importance of digestion by bacteria versus digestion by plant enzymes was still unclear. Goebel (1893) had suggested that plant enzymes dominated the digestion processes when the pitchers were young and the pH was low, but when the pitchers aged, the pH rose and microorganisms became responsible for more of the digestion. Vines (1877) had shown that extracts from young pitcher walls digested protein, but in older pitchers this activity was lost. Hepburn (1918) found that bacteria isolated from the pitchers could digest protein and that the colonies always causes an alkaline reaction.

Three studies in the 1930s filled out our picture of the role of endogenous versus microbial digestion. Stern and Stern (1932) tested the digestive activity of fluid taken from open pitchers with insects present, at different pHs. They found that the fluid generally had two optimal activities, one at a low pH and one nearer to neutral. When they tested digestion of albumin with pitcher extract instead of fluid, they found only one optimum at low pH. Okahara (1933) found that bacterial isolates from pitcher fluid were active against a range of proteins when tested in the pH range 6-8. At pH 3.3, however, none of these isolates degraded protein and most did not even survive. Some fungi isolated from the fluid, however, could still degrade protein at low pH. Zeeuw (1934) showed that if the fluid of open pitchers was kept sterile by use of bromine water and a cotton wool plug, the neutral pH optimum of digestion found by Stern and Stern was lost, suggesting it was derived from bacterial activity, and the low pH digestion was endogenous.

In 1942, Lloyd reviewed digestion in *Nepenthes* and concluded:

> As the matter stands at the present, therefore, the positive evidence that [an acid protease] is secreted by the pitchers of *Nepenthes* is conclusive. That...digestion in the absence of bacteria takes place there seems little doubt, but this cannot yet be said to be completely proven.

This statement marks the transition to the modern era, in which the focus is not whether *Nepenthes* secrete compounds that digest protein, but rather how to isolate, purify and characterize the protease(s) that exist. By this time, several characteristics of nepenthin were known: 1) Its activity is increased by acid conditions (*e.g.,* Gorup-Bensanez, 1874; Gorup-Bensanez and Will, 1876; Vines 1877, 1897; Zeeuw, 1934). 2) It does not degrade readily over time since pitcher extracts retained activity after two months (Vines, 1897). 3) The digestive activity is also unusually stable against heat and alkali (Vines, 1897). 4) The protease acts on a wide variety of substrates (Stern & Stern, 1932).

The Modern Era: 1964-Present

Previously, all the work characterizing the *Nepenthes* peptidase had demonstrated that the enzyme(s) present are endopeptidases, enzymes that cleave the middle of proteins. When endopeptidases have chopped proteins into smaller pieces, an exopeptidase is required to break off amino acids one by one off the short chains. Lüttge (1964b) showed that one kind of exopeptidase, leucine amino peptidase, was present in opened pitchers, but since it was not present in closed pitchers it was unclear if it was the product of contamination. Lüttge (1964b) also showed that digestive activity increased with temperature, peaking around 50ºC or even as high as 60ºC. The level of activity correlated with the Cl^- concentration in the pitcher (Lüttge, 1966), which strengthened the theory that the low pH in pitchers was due to hydrocloric acid (HCl) secretion.

Steckelberg *et al.* (1967) used chromatography to purify the *Nepenthes* protease to homogeneity. The activity of the purified enzyme from three species showed a

strong peak at pH 2.2 and had little activity above pH 4. Nakayama and Amagase (1969) purified the digestive enzyme using gel-filtration and column chromatography and proposed the name Nepenthesin for it. Again, the optimum pH was 2-3 and its heat stability was demonstrated up to temperatures as high as 60ºC. They tested the direct effect of Cl^- ions on the enzyme activity and found that Cl^- had almost no effect up to concentrations of 10^{-3} M, but at concentrations higher than that (10^{-2} M) the effect was strongly inhibitory. Using several peptides of known structure they showed that the enzyme appears to cleave preferentially next to aspartic acid residues in the polypeptide chain with additional action on the carboxyl side of tyrosine and alanine. This was confirmed by Amagase *et al.* (1969). Amagase (1972) used electrophoresis to spread the enzymes in fluid from unopened and opened pitchers in a gel and then stained the gel for protease activity. Four separate bands (presumably each a different protease) were found in opened pitcher fluid. Three of these were also present in the fluid of unopened pitchers. The purified protease preparation, Nepenthesin, showed only one of these bands. Purified protease from *Drosera peltata* also had only one band in a similar location to the *Nepenthes* band, suggesting that the protease may be the same or similar in the two plants. Other characteristics of the *Drosera* protease (*i.e.* pH optimum, heat sensitivity and peptide cleavage patterns) were similar to those found for Nepenthesin.

Jentsch (1972) used repeated chromatography on ion exchange columns to isolate *Nepenthes* protease from sterile, unopened pitchers. He called it Nepenthacin. Lobareva *et al.* (1973) also purified the protease from *Nepenthes* and found that its activity was eliminated by a potent inhibitor (DDE) to the acid proteases of animals and fungi. This suggested that the active center of the enzyme in *Nepenthes* is functionally similar to that of animals and fungi. Takahashi *et al.* (1974) showed that two other potent inhibitors to acid proteases (DAN and pepstatin) also inhibit digestive activity in *Nepenthes*. Tökés *et al.* (1974) reported two proteolytic enzymatic fractions from unopened pitchers of *N. macfarlanei*. The major enzymatic activity appeared to be a protein of 59 kDa molecular weight while the minor fraction was around 21 kDa. No difference was found in opened pitchers, suggesting that no other protease is secreted after maturation. Studies using acid protease inhibitors on the activity of the 59 kDa protein corroborated the findings of Lobareva *et al.* (1973) and Takahashi *et al.* (1974). They also looked at the digestion products of the 21 kDa enzyme and found that it was different from that of Nepenthesin (which they equated with their 59 kDa enzyme). Their conclusion, however, that the 21 kDa enzyme "completes hydrolysis to free amino acids" does not seem to be convincingly demonstrated by the evidence they provide.

The acid protease was again purified by Athauda *et al.* (1998), this time from *N. distillatoria*. Using (presumably open) pitchers collected in the forests of Sri Lanka, they were able to purify about 2 mg of the protease from 30 l of fluid. They used a pepstatin-Sepharose column which specifically purified the protease. The molecular weight of the protease was measured as 45 kDa or 58 kDa by two different techniques, suggesting that it is the same or similar enzyme as the major fraction of Tökés *et al.* (1974). As with previous studies, they found the pH optimum between pH 2.0-3.0, a temperature optimum at 50-60ºC and complete inhibition by pepstatin. Most interestingly, they report that the purified enzyme stains for carbohydrate. Jentsch (1970, 1972; Jentsch *et al.*, 1989) had complained about the difficulty in purifying *Nepenthes* protease due to interference by carbohydrate in the fluid, even in unopened pitchers. The findings of Athauda *et al.* (1998) suggest that Nepenthesin may be glycoprotein; that is to say, a protein modified with bound sugar molecules.

What is really new about Athauda *et al.* (1998) is that, for the first time, they report the amino acid sequence of part of the Nepenthesin molecule. Proteases exist in all life forms as part of an overall process of protein turnover (Ryan & Walker-Simmons, 1981). At the cellular level, organisms use proteases to break down proteins and reutilize them to support life processes. There are several classes of proteases present in plants and animals; Nepenthesin is an acid protease and is very

similar in function and character to the animal protease pepsin produced in the stomach. Athauda *et al.* (1998) state that, despite the functional similarity of Nepenthesin to other acid proteases, it has little homology (similarity of amino acid sequence) to the acid proteases from barley, rice and cardoon flower. In fact, the sequences of other known plant acid proteases show more homology to each other than they do to Nepenthesin. Athauda *et al.* (1998) conclude that Nepenthesin is a unique protease among plant and other acid proteases.

Even with all this evidence, the controversy continues as to whether the digestion in *Nepenthes* is effected by endogenous proteases. Tan and Ng (1997) cite unpublished work by Chia that digestion in young pitchers is accomplished via free radicals (reactive oxygen species such as H_2O_2, OH^-, and superoxide O_2^-) and not by endogenous proteases. Free radicals can digest protein, carbohydrate and lipids. They claim that free radical production increases with the maturity of the pitcher, peaking just after the lid opens, and declines thereafter. After the decline in free radical production, they argue, digestion of prey is effected by bacterial-derived enzymes. Santo *et al.* (1998) used photographic film placed in contact with gland tissues (see Heslop-Harrison & Knox, 1971; Hartmeyer, 1997) from *N. alata* to detect protease activity and claim that these glands do not secrete enzymes. They suggest that insect bacteria may be the primary digestive agent and that the glands appear specialized for nutrient uptake rather than digestion.

Conclusions

One hundred and twenty five years after Hooker first demonstrated the digestive ability of *Nepenthes* fluid, there is still a great deal we do not know about the process. I think the evidence is conclusive that *Nepenthes* produce and secrete an endogenous acid protease. We know that it is an enzyme relatively stable to heat, increased pH, and degradation over time (unlike the mammalian protease pepsin which it otherwise resembles) and that it is effective against a wide range of protein substrates. Specific qualities of this enzyme have led directly to much of the difficulties in its study. It is produced, most likely, only in small quantities; it is associated with carbohydrate and has a relatively low and narrow pH optimum. Studies performed at the ostensibly low pH of, say, 4.0 may still be too high to see significant action of the enzyme; pitchers with fluid pH above the optimum range may appear to be incapable of using Nepenthesin for digestion. It should be kept in mind, however, that the overall pH of pitcher fluid may not accurately reflect the true pH in the region of the glands where digestion and absorption occurs. Much of the confusion concerning *Nepenthes* digestion has revolved around the paradox of how to disentangle the role of any endogenous protease(s) from that of the digestive activity of microbes living in the pitcher fluid. Studying fluid from unopened pitchers may exclude bacteria, but a priori there is no reason to suppose that the enzyme is present or functional in closed pitchers since these are not yet capturing prey (some, but not all, studies have found the enzyme in unopened pitchers). On the other hand, studying digestion with fluid from opened pitchers will always be subject to the criticism that any digestion observed is really a function of microorganismic contamination, unless the plants are studied under sterile conditions *in vitro*.

Several questions about *Nepenthes* digestion are still unanswered. We do not know if the acid protease (nepenthesin) is produced first as a zymogen—an inactive precursor that is activated by low pH and the cleavage by existing protease (as is the case with pepsin). We do not know if there is more than one acid proteases produced by *Nepenthes* or if there are other kinds of proteases produced (such as exopeptidases). Another question that has scarcely been addressed is whether the acid protease(s) are in common to all *Nepenthes* species or if there might be significant differences among species. Is it possible some species forego endogenous production of proteases and let the microbes do all the work? Is the production of functional proteases disrupted in at least some hybrid taxa? Answers to such questions lie in the hands of future scientists or even enthusiastic laymen.

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