Applications of natural deep eutectic solvents to extraction and preservation of biomolecules

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C H A P T E R 5

Stability of enzymes in natural deep eutectic solvents

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Abstract

Combinations of a certain natural small molecules e.g. sugars, amino acids, organic acids and bases in certain ratios can form eutectic mixtures, which are known as natural deep eutectic solvents (NADESs). The NADESs may play a role as alternative media to water for many non-water- or lipid-soluble metabolites in organisms. Not only small molecules but also biopolymers such as DNA, RNA, proteins and polysaccharides were shown to be solubilised by NADESs. It is essential that the integrity of these biopolymers be protected in extreme conditions such as drought or sub-zero temperatures. It was hypothesized that NADESs could preserve DNA, RNA and proteins, keeping them in solution when water is lost by the plant due to drought or water is frozen. In this study, the ability of NADESs to stabilize and preserve macromolecules such as proteins (enzymes) was explored. Enzymes are proteins which can denature easily when exposed to high temperatures and pH changes, requiring specific conditions for optimum activity. Proteases were selected as model enzymes to investigate the interaction between proteins and NADESs. Proteases are found, among others, in carnivorous plants playing an important role in the digestion of the trapped insects. The production of these plant proteases is triggered either by the presence of a prey or by stimulating agents such as chitin and jasmonic acid. These two compounds were used in this study to induce protease activity in *Drosera capensis* L. variety Rubra glands. Bromelain is a protease present in the stem and flesh of pineapple fruit. In this study, the activity of both fresh protease from *Drosera* sap and bromelain-containing pineapple juice were determined. Commercially obtained protease and bromelain were used to test the stabilization and preservation of enzymes in NADESs. For this, both protease and bromelain enzymes were stored and kept in NADESs, and their activity was examined over time. NADESs used in this experiment were Na1 (mannose-glucose-choline chloride-water), Na2 (fructose-sucrose-glucose-water), and Na3 (sucrose-betaine-water). The results showed that the NADESs tested in this study could stabilize the

enzymes preserving their activity as shown by their efficiency with substrates, even after 14 days of storage at room temperature. Based on the results it was concluded that certain NADESs might be candidates for enzyme preservation.

Keywords: Enzyme stabilization, proteases, bromelain, NADESs, preservation.

Introduction

Cell metabolism is a complex network of a wide range of different molecular interactions, e.g. signaling, gene activation, transcription, translation, synthesis, catabolism, transport, and regulation. All cells in living organisms synthesize a huge variety of molecules of diverse masses and polarities, ranging from simple molecules, such as sugars, amino acids or nucleotides to their corresponding polymers e.g. polysaccharides, proteins, DNA and RNA. Proteins are in fact the basis of the cellular machinery, functioning as catalysts, signals, receptors, and transporters. While most proteins have a highly specific function some have a more general function as for example in plants where oxidases and proteases play essential physiological roles. Proteases are also involved in the digestion of food in mammals, and even in carnivorous plants. Enzymatic reactions require very specific conditions such as a determined temperature and pH range and the presence of co-factors. This is because the function of a protein is very much dependent on its configuration and conformation. Thus, changes in the inter- and intracellular conditions of cells may damage the proteins resulting in the loss of activity. This might eventually result in cellular death.

Under extreme conditions, such as drought and cold, resistant organisms appear to form a liquid phase consisting in low-molecular weight organic compounds to store and protect essential macromolecules such as proteins and cellular membranes from decomposition and damage, respectively. In methanogenic organisms (Archaea), cyclic-2,3-bisphosphoglycerate is produced, whilst di-*myo*-inositol-phosphate is found in *Pyrococcus woesei*, *Metanococcus igneus*, and *Pyrococcus furiosus* as thermoprotectants in high temperatures. Di-*O*-β-mannosyl-di-*myo*-inositol-phosphate and di-*myo*-inositol-phosphate are found at supra optimal growth temperatures (8 ^{o}C above optimal growth) in *Thermatoga maritima* and *Thermatoga neapolitana*. In thermophilic bacteria, 2-*O*-β-mannosylglycerate is accumulated as a protectant when submitted to hot thermal and osmotic stress (Rudnick et al. 1990; Martins et al.

1996; Ramos et al. 1997). Mannosylglycerate has been evaluated as a cryopreservation agent for the freeze-drying of enzymes isolated from mesophilic (moderate temperature), thermophilic, and hyper thermophilic resources. The results indicated that mannosylglycerate effectively stabilizes enzymes (Ramos et al. 1997). Trehalose is also effective for the preservation of dehydrated enzymes without loss of activity after prolonged storage (Colaco et al. 1992).

The existence of a third type of liquid phase in nature (cells) has been hypothesized by Choi et al. (2011). This phase could have various functions that cannot be explained in an aqueous or lipid phase. These hypothetical solvents were named natural deep eutectic solvents (NADESs). They are composed of various compounds mixed in specific molar ratios, e.g. sugars, polyalcohols, amino acids, organic acids, and organic bases (Dai et al. 2013; Paiva et al. 2014; Vanda et al. 2018). It has been suggested that NADESs play an important role in the survival of plants during drought, desiccation or cold conditions as well as constituting a media for the biosynthesis and storage of non-water soluble metabolites. Among other properties, may be able to stabilize enzymes in nature. Much research has been reported on enzymatic activities in synthetic ionic liquids or deep eutectic solvents. Park and Kazlauskas (2003) reported that hydrolase and oxidoreductase types of enzymes retain their activity in ionic liquids, and Ninomiya et al. (2012) showed that ionic liquids can be applied in the enzymatic hydrolysis of cellulose. Deep eutectic solvents are also suited for enzymatic reactions, for example in epoxide-hydrolysis (Lindberg et al. 2010), transesterification of cross-linked subtilisin and α-chymotrypsin (Zhao and Baker, 2013). Other examples of recent uses of NADESs as media for various enzymatic reactions, are biodiesel synthesis (Durand et al. 2013), saccharification of rice straw (Kumar et al. 2016), and whole-cell biocatalysis of isoeugenol to vanillin in *Lysinibacillus fusiformis* (Yang et al. 2017). The use of NADESs as a stabilizing solvent was reported for chondroitinases ABCI, a clinical enzyme for treating spinal lesions (Daneshjou

et al. 2017).

Insect-eating plants are very interesting organisms. Either alone or in symbiosis with microorganisms, the plant is able to use insects as its energy source. The plant exudate attracts the prey. Once the prey is trapped, digestive enzymes are produced and released into the exudate. These enzymes, mostly proteases, are active for days, digesting the prey. Protease production can also be induced by simulating the presence of a prey with agents such as chitin, gelatin, or salicylic acid, which mimic the presence of a prey (Matušíková et al. 2005). In this study, the stimulating effect of jasmonic acid was tested as it acts as a plant hormone. There are two systems for attracting the prey. One of them occurs in beaker-like flowers that have a liquid exudate that contains the enzymes that digest the insect. If pure water were the basis of the liquid exudate, its stability could be problematic, due to the evaporation of the water when exposed to the high ambient temperatures or to the sunshine. However, were the exudate a water-containing NADESs, this would not occur.

The other mechanism for trapping insects is with a glue on the surface of leaves. For example, in *Drosera* species, the glue must maintain its liquid state on the leaves in all conditions, pointing to the option of this exudate being a NADESs. Both types of carnivorous plants might thus combine proteases as digestive enzymes and NADESs components to solubilise the enzymes and possibly the prey. This is an interesting model for further studies.

Proteases have been investigated by researchers for centuries due to their important roles in biological systems, especially related to digestive purposes, extracellular modelling, protein-protein interaction, processing of cellular information, and many more. Proteases are present in all living organisms, having important physiological functions such as the digestion of proteins and more specifically, in regulated processes such as blood coagulation, fibrinolysis, and transport of secretory proteins across membranes (Neurath 1984).

Proteases can be found in bacteria, algae, viruses, plants, and animals. In carnivorous plants, proteases break down the digestible material from the prey

to smaller molecules that can be absorbed by the plants. In *Drosera* plants, proteases are produced in the glandular hairs, where the prey (an insect usually) is captured by the sticky mucilage produced by these glands in the tentacles of the leaves. After the insect is trapped, the leaf will roll-up, enveloping the prey, and proteolytic enzymes will subsequently be released for its digestion (Król et al. 2011; Poppinga et al. 2010); Nakamura et al. 2013). Several enzymes have been detected in *Drosera* e.g. proteases, acid phosphatase, chitinase, peroxidase, and esterase. The process of digestion and absorption of the prey takes approximately 48 hours (Amagase 1972; Matušíková et al. 2005; Libantová et al. 2009; Król et al. 2011). The sap from the fruits of A*nanas comosus* (L.) Merr. was used in our study, as a model. Pineapple fruit are well-known to contain bromelain, a protease which has various medicinal and food applications. Bromelain is used as an anti-inflammatory agent, and in recent years, researchers have also investigated its possible anti-cancer activity. Bromelain has been proved to stimulate autophagy activity in mammary carcinoma cells (Bhui et al. 2010). It shows anti-proliferative and pro-apoptotic effects in colorectal carcinoma cells (Romano et al. 2014), and significantly inhibits cell proliferation in human gastrointestinal carcinoma cell lines. In combination with *N*-acetylcysteine the effect was found to be increased (Amini et al. 2014). Bromelain was thus used as a model for proteases in our studies.

To investigate the possible role of NADESs, particularly in enzyme activity, sugar-based NADESs were employed. Several sugars, including mannose, galactose, xylose, arabinose, and glucuronic acid have been detected in *Drosera capensis* exudates by Gowda et al. (Gowda et al. 1982; 1983), and our group has detected acetoacetic acid, acetic acid, *myo*-inositol, glucose, sucrose, lactic acid using ^{1}H NMR, and mannose, fructose, arabinose, galactose, and glucuronic acid using GCMS. All these components are possible NADESs constituents. If proteases were dissolved in NADESs, these could contribute to capture the prey because of their stickiness, and also provide suitable conditions for the enzymatic reactions. We thus hypothesized that given the chemical

composition of both pineapple sap or the sap of carnivorous plants, these could actually be natural deep eutectic solvents (NADESs) and could stabilize enzymes such as proteases in nature.

NADESs are a new generation of natural ionic liquids. Ionic liquids have been a hot topic in research when attempting to reduce or replace toxic organic solvents used in chemical syntheses (Abbot et al. 2004). There are also numerous examples of their use as media for enzymatic reactions such as the enzymatic transesterification of triglyceride with methanol for biodiesel (Zhao and Baker 2013), the hydrolysis of cellulose (Ninomiya et al. 2012), the lipase-catalyzed epoxidation and Baeyer-Villiger oxidation (Kotlewska et al. 2011). However, many synthetic components of ionic liquids are actually relatively toxic (Zhao et al. 2007; Romero et al. 2008). NADESs on the other hand, can be considered as "green" solvents because their components are found commonly in our food, their production is sustainable and they are easily biodegradable. Toxicity and cytotoxicity of some NADESs have been assessed, showing that NADESs have a lower toxicity and cytotoxicity profile even compared to DES (Mbous et al. 2017; Hayyan et al. 2013).

Thus, NADESs are promising alternative solvents to the synthetic ILs. Their application to enzymatic reactions however, has scarcely been evaluated. In the search for evidence of the role of NADESs as a third liquid phase in cells for the storage of enzymes, for example, or in the regulation of enzyme activity we considered that the insect-trapping plant system could be an interesting target for this.

The aim of this study was to determine if NADESs plays any role in stabilizing and preserving proteases enzymes as shown in the prey-digesting process in *Drosera capensis* and in pineapple sap. To answer this question, the activity of protease from different parts of the prey-catching leaves, and the activity of bromelain from pineapple juice was determined.

The commercially available *Streptomyces griseus* protease and the pineapple protease, bromelain, were used as models in the study of activity and

stability in sugar-based NADESs applications. The stabilizing and activity-preservative capacity of NADESs as a media for enzymes were studied to prove that they might function as a third liquid phase in cells for the storage of enzymes and in the regulation of enzyme activity.

Materials and Methods

Plant materials

Drosera capensis L. variety Rubra was collected at Hortus Botanicus Leiden and *Ananas comosus* (L) Merr. fruits were purchased from a local store in Leiden during spring time.

Chemicals

D-fructose, D-glucose monohydrate, and sucrose were purchased from Boom B.V. (Meppel, The Netherlands), and D-mannose, choline chloride, betaine, and jasmonic acid from Sigma Aldrich (The Netherlands). Protease (isolated from *Streptomyces griseus*) and bromelain (from pineapple) were purchased from Sigma Aldrich (catalog number of P8811 and B4882, respectively). A protease-kit was acquired from Pierce Biotechnology (Thermo Fisher Scientific).

Determination of protease activity in Drosera materials

The protease production in the tentacles of *D. capensis* L. Rubra was induced by spreading 0.5-1.0 mg of chitin powders on the surface of leaves for 24 hours (Matusikova et al. 2005). Leaves without any treatment were used as a negative control. Jasmonic acid, a plant hormone, was used as a positive control by placing one drop of jasmonic acid solution (10 mg/mL in ethanol 40% v/v) on the leaf surface. After 24 hours, the remaining inducer was removed by gently flicking the leaves. Samples of leaves, hairs (tentacles) and exudate were taken from three leaves of *D. capensis* L. Rubra plants, (n = 3) collected separately in 2 mL-micro-tubes. After harvesting exudates (approximately 60

µL), 100 µL of glycine-HCL buffer was added to each sample to have a sufficient volume for measurement. After collecting the exudates, the tentacles were harvested after rinsing twice with buffer solution to remove any residual exudate, and subsequently removed from the leaves using a scalpel. The tentacle- and leaf materials (10 mg) were ground and extracted with 150 µL buffer. These buffer extracts were kept for further experiments. The presence of protease in the samples was analyzed following a method described by Bohak (1970) and Matušíková et al. (2005). A volume of 150 µl of each sample was incubated with 150 µl of substrate (2% bovine serum albumin (BSA) in 200 mM glycine-HCl pH 3.0) at 37ºC for one hour after which trichloroacetic acid (450 µL) was added to stop the reaction. Samples were left on ice for 10 min and subsequently centrifuged at 20.000 g for 10 min. The absorbance of the supernatant was measured at 280 nm with reference to the blank (buffer only). One unit of enzyme activity is defined as the amount of enzyme required to cause a unit increase in absorbance at 280 nm (Bohak 1970; Matusikova et al. 2005).

Determination of protease activity after storage of the enzyme in natural deep eutectic solvents (NADESs)

In this experiment, the ability of some NADESs to dissolve and stabilize protease (P-8811) from *Streptomyces griseus, (*Sigma Aldrich, St. Louis, MO, USA) was determined by measuring the activity of the enzyme after storage in different NADESs during 2 weeks (0, 1, 7, and 14 days). The NADESs chosen in this study contained some compounds that were found in *Drosera* exudate and in pineapple sap, such as mannose, sucrose, glucose, and fructose, thus resembling the NADESs proposed to exist in nature. The NADESs were prepared as described by Dai et al. (2013), with a water content that varied between 30%, 40%, and 50% (w/w) for each NADESs. The composition of the tested NADESs were as follows: Na1 (mannose-glucose-choline chloride, 1:2:1, mol/mol), Na2 (fructose-sucrose-glucose, 1:1:1, mol/mol), Na3 (sucrose-

betaine, 1:2, mol/mol), buffer was used as positive control.

The test solutions were prepared by mixing 1 ml of each NADESs with 1 mg of protease in a 2 mL-plastic microtube. The mixtures were homogenized using a vortex until complete dissolution of the enzyme in the NADESs. If necessary, mixtures were additionally sonicated for 2-3 minutes. The mixtures were subsequently stored at room temperature (RT) in the day light for 14 days. Sampling followed a time course (0, 1, 7 and 14 days), and the enzyme activity was evaluated using the previously described method.

Determination of protease activity after storage in low-water containing NADESs

This experiment aimed to investigate the effect of low-water containing NADESs on the preservation of protease activity. The enzyme (1 mg) was first dissolved in a water-containing NADESs (40% water, w/w), after which the solutions were freeze-dried for 24 hs to eliminate the water. Sampling of this enzyme-NADESs solution was done by weighing, instead of pipetting, due to the high viscosity of the mixture. Protease activity of the sample was determined using the same protocol as described previously on days 0, 1, 5, and 7.

Determination of bromelain activity of pineapple juice

Pineapple juice was collected by expression of the fresh fruit, and directly used for bromelain activity test using the protease activity test.

Metabolic profiling of pineapple juice by ^{1}H NMR

Pineapple was peeled, cut in small pieces and crushed to obtain juice that was then freeze-dried for 48 hours. The dried juice (20 mg DW) was dissolved in 500 µl of deuterated solvent (D_2O KH_2PO4 buffer 1 M, pH 6) with 0.005% TMSP (w/v) (Kim et al. 2010). The samples were ultra-sonicated for 15 minutes followed by centrifugation at 13.000 rpm for 10 minutes. An aliquot of 300 µL of each sample was transferred to a 3-mm NMR tube and their ^{1}H-NMR

spectra were recorded at 25 ºC on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany). The resulting spectra were manually phased and baseline corrected, and calibrated to TMSP at 0.0 ppm using Topspin (Bruker).

Determination of bromelain activity after the storage of the enzyme in natural deep eutectic solvents (NADESs)

Solutions containing 1 mg of bromelain powder (B4882 from pineapple stem, Sigma Aldrich) in 1 ml of NADESs with different water concentrations (30%, 40%, and 50% w/w) were prepared. The NADESs used for this experiment were the same as those used for the protease analysis, that is: Na1 (mannose-glucose-choline chloride, 1:2:1, mol/mol), Na2 (fructose-sucrose-glucose, 1:1:1, mol/mol), Na3 (sucrose-betaine, 1:2, mol/mol). The samples were stored at room temperature in the day light, and tested on day 0, 1, 7 and 14 after preparation for their bromelain activity using the Protease Assay Kit (Thermo-scientific USA).

Determination of bromelain activity after the storage of the enzyme in low water-containing NADESs

This experiment was conducted to examine the effect of low water-containing NADESs on the preservation of bromelain enzymatic activity. For this, 1 mg of bromelain powder was dissolved in 1 ml of the different NADESs (containing 40% water), after which the solutions were freeze-dried for 24 hs to eliminate the water. The samples were stored at room temperature in day light, and collected on day 0, 1, 5, and 7 days after freeze-drying to determine bromelain activity as described previously.

Microscopic Observation

This experiment aimed at examining a possible effect of microorganism growth on the enzyme-NADESs mixture observed in the samples. Mixtures of protease-NADESs and bromelain-NADESs were incubated at 37 ºC for seven

days, using a buffer-enzyme mixture as a positive control and NADESs and buffer samples without enzyme as controls. On day 3 and day 7, 300 uL of all samples were pipetted and transferred separately in 8-well plates and observed under a Zeiss Observer laser scanning microscope equipped with fluorescence filters at x63.

Results and Discussion

This study was carried out to analyze the preservation and stabilization of proteases enzymes in NADESs. Protease activity in the exudate of *D. capensis* L. Rubra after induction with chitin and jasmonic acid, as a model for the prey-digesting processes was determined, as well as bromelain activity in pineapple sap. Additionally, the stability and activity of *Streptomyces griseus* protease (SG-protease) and bromelain (from *Ananas comosus* (L.) Merr.) stored in several NADESs were also examined.

For this, the leaves of the plant were treated with chitin or jasmonic acid (JA) to mimic the prey-catching process, allowing the study of the following digestion of the prey. The levels of protease activity in the *Drosera* exudates and tentacles after a chitin induction were similar to the negative controls (without chitin induction), whilst in the samples treated with JA, the levels were much higher. No protease activity was detected in the leaf material (Figure 1).

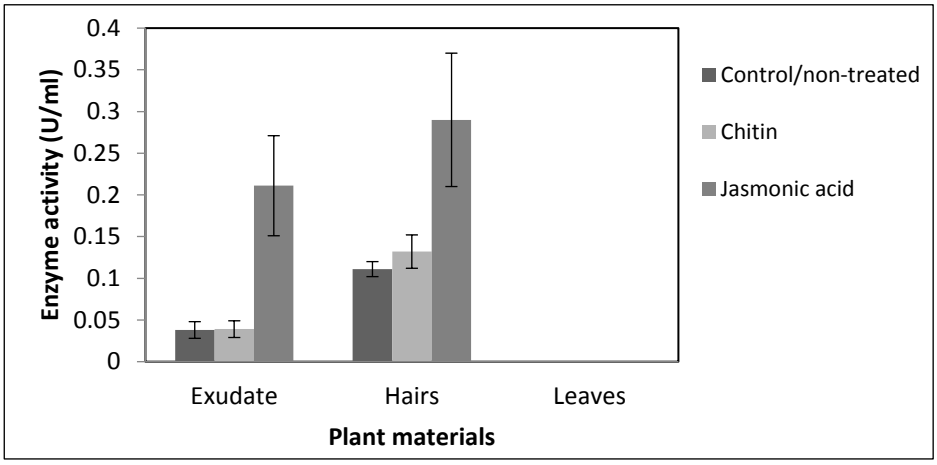

Figure 1. Protease activity of *Drosera capensis* L. variety Rubra leaves exudate induced with chitin and jasmonic acid.

Jasmonic acid is a plant hormone which is involved in the bending movement of the tentacles of *Drosera*, that signals the initiation of the prey-digesting stage (Nakamura et al. 2013). When the plant is induced with insect or insect-derived oral secretion, JA production increases, inducing both the plant defense system against wounding and the prey-digesting process (Mithöfer et al. 2014). Thus, the presence of a prey on a leaf stimulates leaf-bending to fully trap the prey and allow the subsequently produced and excreted enzyme to digest the prey. When JA is used as an induction agent, it triggers the transcription of the gene encoding proteases (Herrmann et al. 1989; Mueller 1997). The JA treatment is thus a useful model system to study the prey-digesting process. The next question to be answered was whether NADESs could play a role in the digesting process itself. The role could be two-fold, i.e., to act as a stable medium for prey-digestion, stabilizing the enzymes and ensuring that the medium does not dry up under the ambient conditions including strong sunlight and heat. The other function is to dissolve the prey, particularly to solubilize the macromolecules, making them available for the digesting process.

To study the stability of proteases, we used two commercially available

proteases, one from *Streptomyces griseus* (SG-protease) and bromelain from pineapple fruit. To validate our protease assay we determined the bromelain in fresh pineapple juice (Figure 2). The ^{1}HNMR spectra of the juice, revealed the presence of sucrose-glucose-fructose in about equal amounts. This is, clearly, the basis of a NADESs in the otherwise aqueous juice.

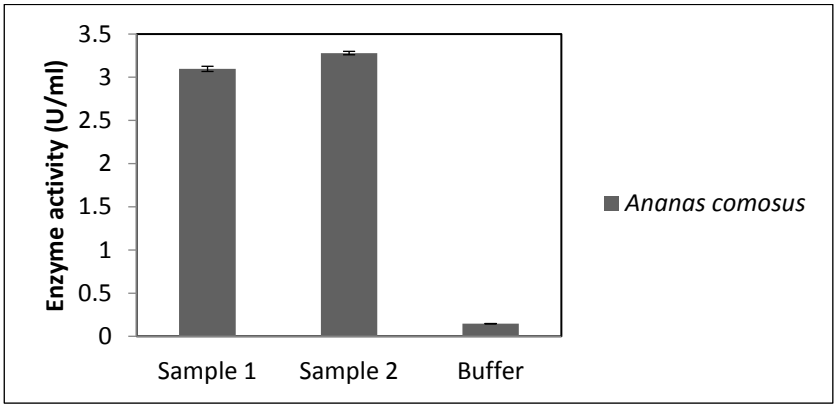

Figure 2. Bromelain Activity in *Ananas comosus* L. Merr. juice.

The SG-protease, dissolved in all three tested NADESs with different levels of water, was relatively stable after 14 days of storage at room temperature and in the daylight, showing high activity (Figure 3). In the buffer solution of the protease, enzymatic activity was significantly higher than in NADESs Na1 and Na3 on day 0 and 1. However, no activity was detected after 14 days.

Interestingly NADESs Na1 (30%, 40% water) and Na3 (50% water) showed a significantly strong increase in activity after day 1 ($p<0.05$), and stayed high until day 14. Comparing the three NADESs, the Na2 type show highest activities. The more concentrated Na3 type seems to have lowest activity, though with 50% of water after 1 day the activity raises to similar levels as the others.

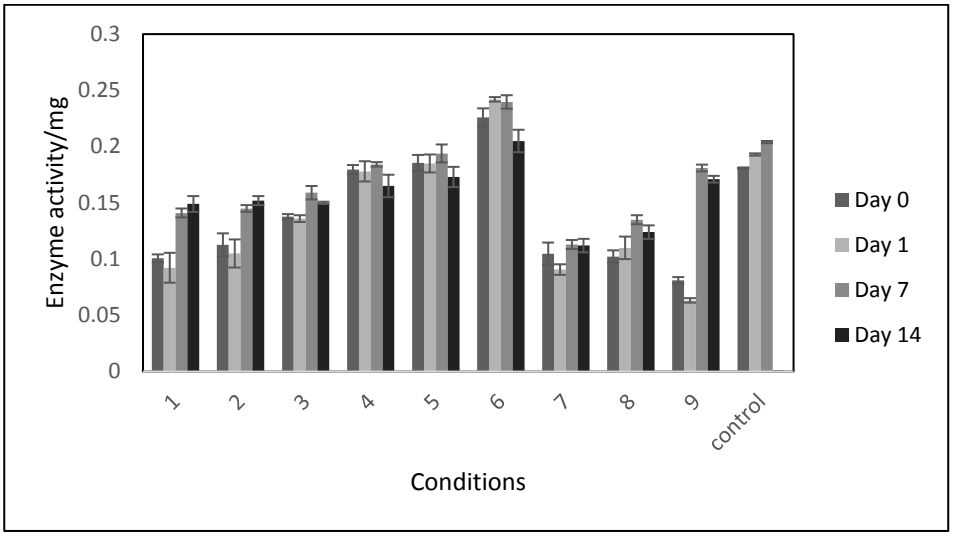

Figure 3. The effect of water on the preservation of SG-protease in three NADESs with different water content stored under ambient conditions in daylight. 1-3: Na1 (mannose-glucose-choline chloride, 1:2:1, mol/mol) with 30%, 40%, 50% water (w/w), 4-6: Na2 (fructose-glucose-sucrose, 1:1:1, mol/mol) with 30%, 40%, 50% water (w/w), 7-9: Na3 (sucrose-betaine, 1:2, mol/mol) with 30%, 40%, 50% water (w/w).

In order to determine whether the presence/absence of water in NADESs influenced the stability of the enzyme, the enzyme was dissolved in the NADESs with 40% water and then freeze-dried to remove the water. After freeze-drying, the enzyme-NADESs solutions became very viscous and sticky, except for Na3, which still had a low viscosity. The most viscous and sticky solution was Na2. The freeze-dried Na3 preparation had the highest activity among the NADESs and was still active at day 7. The lowest activity was observed for the Na2 solution of SG-protease, which had the most viscous consistency. The level of protease activity in all NADESs was lower than that determined for the same NADESs containing water (Figure 4). The highest activity was 0.127 U/mg enzyme as indicated in Na3. It was thus concluded that the higher concentration of NADESs ingredients could affect the activity in the protease assay.

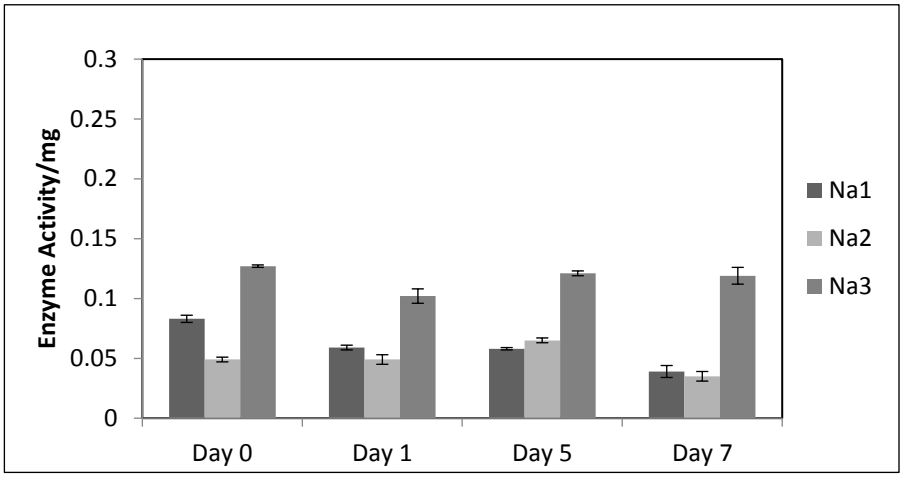

Figure 4. The stability of freeze-dried low water containing NADESs with SG-protease stored at room temperature in the light during 7 days, as measured by enzymatic activity.

^{1}H-NMR-based metabolomics was performed on dried pineapple juice. The ^{1}H-NMR showed that the main compounds were fructose {4.16 (d), 4.01 (d), 4.0 (t), 3.7 (d) ppm}, sucrose {5.42 (d), 3.7 (s), 3.6 (s), 3.5 (dd) and 3.4 (t) ppm}, and glucose {5.23 (d), 4.6 (d), 3.9 (dd), 3.8 (m), 3.2 (m) ppm} (Figure 5) in approximately equal molar quantities, i.e. the basis of a NADES.

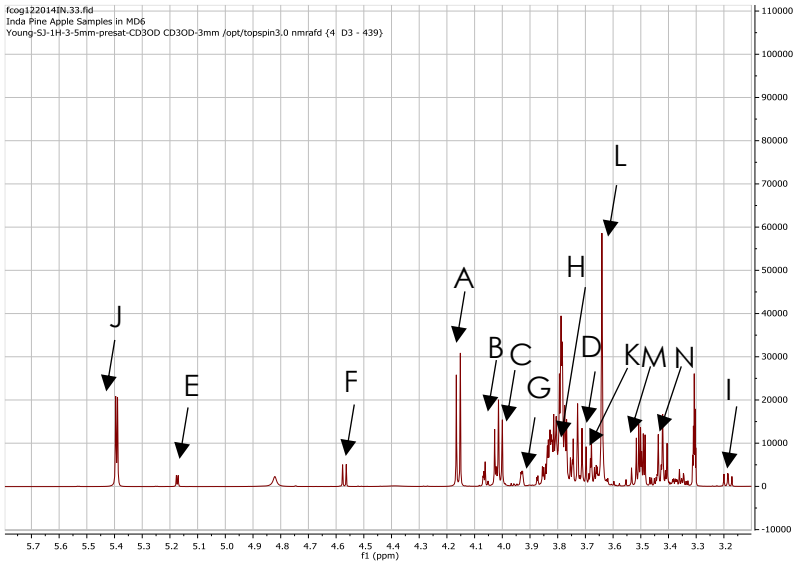

Figure 5. [1]H NMR spectra of pineapple juice. Chemical shifts for fructose are at A. 4.16 (d), B. 4.01 (d), C. 4.0 (t), D. 3.7 (d) ppm; glucose at E. 5.23 (d), F. 4.59 (d), G. 3.9 (dd), H. 3.8 (m), I. 3.2 (m) ppm; and sucrose at J. 5.42 (d), K. 3.7 (s), L. 3.6 (s), M. 3.5 (dd) and N. 3.4 (t) ppm

Commercial bromelain was dissolved in water-containing NADESs and the activity of the enzyme was measured at defined time points during a 14-day storage period at room temperature in the daylight. Activity was measured with a Protease Assay Kit (Thermo-scientific, USA). The highest bromelain activity was detected for reactions in buffer solutions and Na1 with 50% of water on day 0, decreasing significantly throughout day 1-14 ($p<0.05$). For 30% of water it was Na2 that gave the highest activity. In Na1 30%, day 0 and 1 exhibited high activity, decreasing significantly at days 7 and 14 ($p<0.05$). In Na3, the activity was also the highest on day 0, decreasing significantly after day 1. For all NADESs, the enzymatic activity decreased during storage, but in the case of the buffer no activity at all was observed at day 14 (Figure 6). It is important to note that the buffer solution was heavily contaminated with microorganisms. To measure the effect of a concentrated NADESs, water-containing solutions of bromelain in the three types of NADESs were freeze-dried. The enzyme activity in these low water-containing NADESs solutions was monitored during a 7-day

storage period in ambient conditions and exposed to daylight. At all measuring points, Na3 solutions exhibited the highest activity, whereas Na2 showed the lowest activity at day 0 and no activity on day 7 (Figure 7).

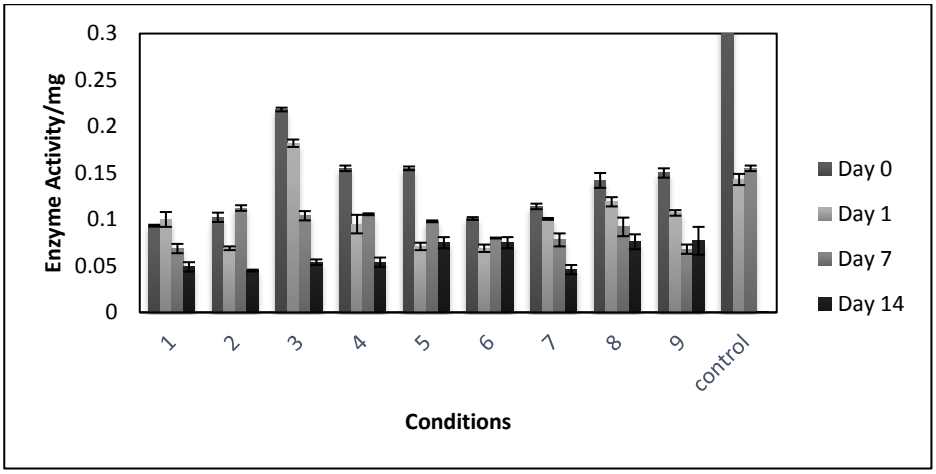

Figure 6. The effect of water on the preservation of bromelain enzymatic activity in three NADESs solutions vs an aqueous solution of bromelain. 1-3: Na1 (mannose-glucose-choline chloride, 1:2:1, mol/mol) with 30%, 40%, 50% water (w/w), 4-6: Na2 (fructose-glucose-sucrose, 1:1:1, mol/mol) with 30%, 40%, 50% water (w/w), 7-9: Na3 (sucrose-betaine, 1:2, mol/mol) with 30%, 40%, 50% water (w/w).

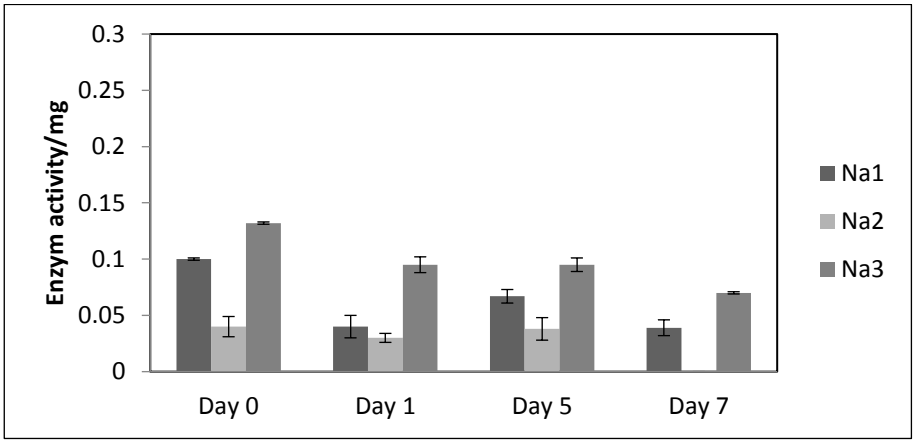

Figure 7. The ability of some low water-containing NADESs to stabilize bromelain stored at room temperature in daylight during a time course, determined by it enzymatic activity.

Microscopy

Microscopy was used to determine microbial growth in NADESs-enzyme and buffer-enzyme solutions. The solutions of SG-protease were analysed on day 3 and 7 after their preparation. Microscopic observation of all the NADESs-protease mixtures on day 3 and 7 showed the absence of any microbial growth. However, a high bacterial count was registered in the buffer-protease solution on day 3 and after 7 days, the count had increased significantly, and fungi and yeast were also detected.

Microscopy of all NADESs-bromelain mixtures did not show any microorganism growth on day 3. However, a small number of bacteria were observed in Na1 with 30% water on day 7. On day 7, bacteria and also fungi were observed in all NADESs-bromelain solutions containing 50% water, while in buffer solutions, there were large amounts of microorganisms including bacteria, fungi, and yeast.

No microorganisms were detected in NADESs containing no enzymes, only some possible microorganism debris was detected in Na1 and Na2. Some fungi were detected in the buffer solutions containing enzymes, proving that this buffer was a favourable media for microbial growth (Figure 8-9). It also shows that enzymes in NADESs provided a good source of nutrients for microorganism growth.

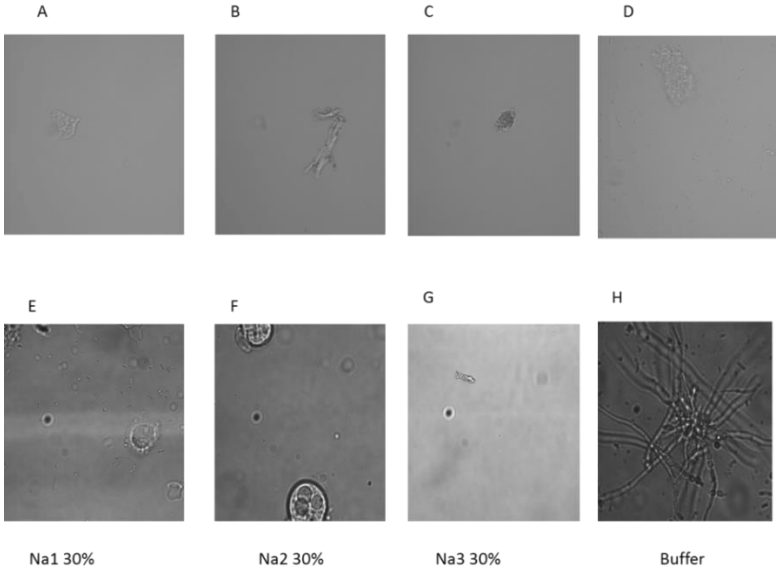

Figure 8. Microscopic observation of SG-protease and bromelain dissolved in three NADESs containing 30% of water (w/w) stored at room temperature. A-D: day 3. E-H: day 7. A: SG-Protease in Na1, B: SG-protease in Na2, C: SG-protease in Na3, D: SG-protease in buffer, E: bromelain in Na1, F: bromelain in Na2, G: bromelain in Na3, H: bromelain in buffer.

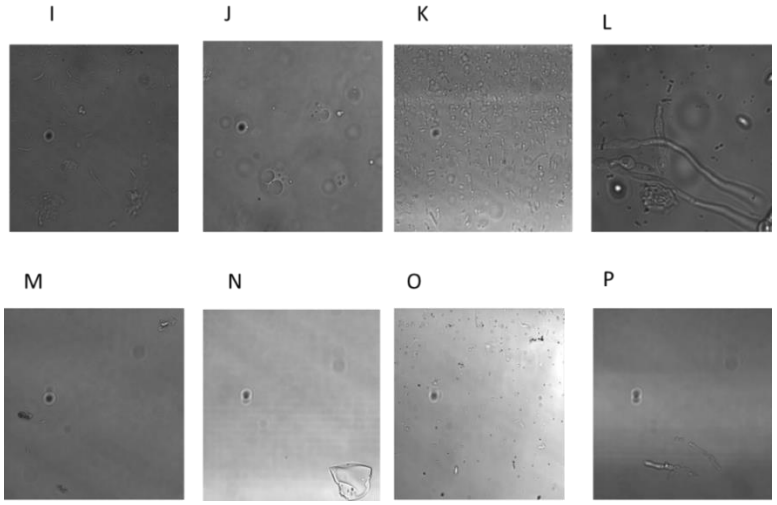

Figure 9. Microscopic observation of bromelain dissolved in three NADESs containing 50% of water (w/w) stored at room temperature on day 7. I: bromelain in Na1, J: bromelain in Na2, K: bromelain in Na3, L: bromelain in buffer. As control, NADESs with 50% water (w/w) without enzyme, M: Na1, N: Na2, O: Na3, P: buffer.

Conclusions

Protease enzymes are produced by plants for specific functions, e.g. in catalysis reactions, as receptors, in degradation processes, and other cellular metabolisms. To evaluate the potential role of NADESs in preserving and stabilizing proteases enzymes, the stability and activity of two known proteases in NADESs solutions was studied.

All NADESs composed of mannose-glucose-choline chloride-water (Na1), fructose-sucrose-glucose-water (Na2) and sucrose-betaine-water (Na3) with different percentages of water stabilized SG-protease. The highest activity was found in Na2 with 50% of water. Bromelain showed the best activity in Na1 with 50% of water. The SG-protease appeared to be more stable in NADESs than bromelain, since this enzyme lost more than 50% of its activity in 14 days. All NADESs performed better in stabilizing the enzymes than the conventional buffer tested, possibly due to the almost inexistent or total absence of microorganisms in the case of NADESs, whereas the buffer solution showed a high microorganism load after 14 days and the enzymes were totally inactive.

The results do thus point to a stabilizing effect and preservation of enzymatic activity in water-diluted NADESs supporting the hypothesis of the role of NADESs in plants. Further experiments with the native enzymes of the plant in an exudate-mimicking NADESs to evaluate the role of NADESs in other organisms are needed to further prove this hypothesis.

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