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Enzyme activity determination using ultrasound

M J Holmes[1], **T Southworth, N G Watson and M J W Povey.**
The School of Food Science and Nutrition. The University of Leeds,Woodhouse Lane, Leeds LS2 9JT, UK

E-mail: prcmjh@leeds.ac.uk

Abstract. Here are presented the results of a novel approach to the measurement of enzyme reaction rates in which ultrasound velocity measurement is used. Our results show enzyme activity is observable, in the acoustic context, and that furthermore this offers the potential to estimate the rate of reaction over different substrate concentrations and temperatures. Findings are corroborated with optical microscopy and rheological measurements. Ultrasound velocity measurement can be performed without the need for aliquot extraction and offers an efficient, non-invasive and dynamic method to monitor enzyme activity.

1. Introduction

Enzymes are generally globular proteins that are highly selective biological catalysts which accelerate both the rate and specificity of metabolic reactions. Highly specific, they only react with particular substances, known as the subtrate which in turn is transformed into product molecules. The reactions may occur in several ways but all lower the Gibbs free energy [1]. It is thought that approximately 25,000 enzymes exist and the most common way to classify enzymes relates to type of reaction they catalyse [2, 3, 4]. These reactions are important for various uses within the food, chemical, biofuel and medicinal industries. For example, in the food industry, a mixture of pectinases and amylases are used to extract and clarify fruit juice [5]. In this investigation α-amylase, and diastase (α-β-amylase) will be under consideration. Without the presence or addition of of enzymes, reactions would not only progress at a much slower rate, but some may not occur at all. The measurement or estimation of the rate of reaction, known as the enzyme kinetics, can be calculated using a variety of assays and techniques, for example spectrophotometric, fluorometric or chromatographic assays.

There are various factors governing enzyme reaction rates which must be considered when determing enzyme kinetics. Examples being pH, temperature, salt concentration [6] and the concentration of both enzyme and substrate. Typically an enzyme – substrate reaction can be expressed using the Michaelis – Menten equation where the maximum rate of reaction is measured, known as V_{max}, and the concentration of substrate at the midway point of the reaction is measured, known as K_m (often refered to as the Michaelis constant) [4, 7, 8]. Different forms of biochemical analysis are conducted in order to determine the Michaelis – Menten rate constants. Techniques used to determine reaction rates, such as spectrophotometry, may have certain issues that must be considered in order to accurately determine rate reactions. For example, Bisswanger (2004) describes the use of ultraviolet spectroscopy to detect light absorption of a substrate at a particular wavelength; however, absorption at the same wavelength for the product formed was also noted thus creating inaccuracies in the substrate-product kinetics [2].

[1] To whom any correspondence should be addressed.

Background knowledge of the nature of the enzyme and the assay being used is recommended, creating difficulties for those who are less experienced in the field. Analysis techniques can be time-consuming, expensive and invasive with the requirement of aliquots. Discontinuous assays, are labour intensive and due to a 'stop and sample' methodology they sometimes will provide an estimation of the kinetics rather than an accurate measurement [9]. In some cases, techniques such as titrimetric methods are described as not being sufficeintly sensitive enough for use, conversely use of radiochemical methods, although sensitive, are not desirable due to the handling of radioactive materials [10]. Additionally, the rate of reaction is typically calculated using the initial rate, rather than the overall reaction. This can create inaccuracies of its own, with some studies describing fluctuating catalytic rates [11] and issues relating to analysis of only the initial reaction rate [12].

This paper describes how using ultrasonic techniques can offer an alternative method to analyse enzyme kinetics whilst overcoming some of the issues with traditional methods mentioned above. Ultrasound offers a *real-time* method of analysis whereby the dynamics of both enzyme and substrate can be monitored removing the need to prepare and examine several aliquots. The process is non-invasive, non-destructive, may be conducted on optically opaque materials and measurements may automatically collected throughout the reaction minimising process and measurement uncertainties, for example in extraction and sample preparation required for further analyses.

By definition, ultrasonic waves have frequencies exceeding the upper limit of the normal human audible range, being approximately 16 kHz and consist of longitudinal pressure waves which oscillate particles in gases, liquids and solids in the direction parallel to the wave propagation and shear waves which correspond to shear deformations of the material. Ultrasound is now routinely used in many applications including material testing, medical imaging and particle sizing and enables characterisation of the mechanical properties of the medium, e.g. elastic moduli, density, viscosity and phase state [13, 14]. Ultrasonic techniques have become increasingly beneficial for the characterisation of compounds and has been demonstrated to discern molecular structures of compounds [15]. In particular, within the food industry the ability to classify foods [16], assess quality [17], and analyse the composition of food components during processing or over shelf life [18]. It is a technique that is non-invasive, non-destructive and can penetrate opaque materials. Consequently, detailed analysis of a variety of foods can be quickly collected, providing real – time information. A greater understanding of textural and rheological properties can be gained [19, 20], and in relation to this paper, the measurement of processes and reactions through change in viso-elastic density in starch samples are investigated as a novel approach.

Ultrasonic measurements typically consist of the speed of sound v [ms^{-1}] a wave will propagate through a medium and the associated attenuation α [dB MHz^{-1} cm^{-1}] in the incident wave intensity. Under the assumptions of negligible thermo-acoustic losses, the acoustic parameters may be related to the complex elastic modulus $M = M' + iM''$ of the medium where;

$$M' = \rho v^2 \tag{1}$$

$$M'' = \frac{2\rho\alpha v^3}{\omega} \tag{2}$$

Here, ρ is the sample density (kg m^{-3}) and ω the angular frequency (rads s^{-1}) of the wave. The relationships to the conventional rheological parameters of bulk and shear moduli K and G respectively (having the units of Pascals Pa), are as follows;

$$M = K + \frac{4}{3}G \tag{3}$$

where,

$$G = G' + iG'' = \frac{\sigma_0}{\varepsilon_0}\,(\cos\delta + i\sin\delta) \tag{4}$$

During rheological measurements an applied strain amplitude ε_o generates a stress response σ_o with phase lag δ between the stress-strain oscillations. In equation (4), G' is referred to as the storage modulus and G'' the loss modulus [13].

In hydrogels, the bulk moduli K is of the order GPa and is dominated by the water phase which has a shear modulus in the order 10^{-5} Pa and so any shear waves are evanescent. Primary contribution toward the shear modulus manifests through the gel network and is of the order kPa. On this basis and upon examination of equations (1), (3) and (4) one can verify that the speed of sound in liquids is dominated by the bulk modulus giving rise to the approximation commonly quoted in equation (5);

$$v = \sqrt{\frac{K}{\rho}} = \sqrt{\frac{1}{\kappa\rho}} \tag{5}$$

where κ is the bulk compressibility. In solids, since shear waves may be supported in the medium, the corresponding relationships are;

$$c_L = \sqrt{\frac{K + \frac{4}{3}G}{\rho}} \tag{6}$$

$$c_s = \sqrt{\frac{G}{\rho}} \tag{7}$$

where c_L is the associated longitudinal or bulk wave speed and c_s the shear wave speed and of the same order of magnitude with the latter typically reduced by a factor 2-4 [14]. In gels the contribution of the shear storage G' modulus toward the velocity is minimal but in the current ultrasonic context is measurable and offers the capacity to monitor the enzyme action upon the substrate gel network.

Ultrasound is commonly used to study the gelatinisation of starch [21, 22, 23]. In the presence of heat and water, starch undergoes a series of changes leading to gelatinisation. Intermolecular bonds are broken down, allowing hydrogen bonding sites to interact with water. Water is able to diffuse into the armorphous spaces within starch granules causing swelling. This swelling breaks internal structures and granules split. A three-dimensional network forms via starch cross-linking which causes the solution to thicken and form a gel. The temperature of gelatinisation is specific to the type of starch. Gelatinisation temperatures are on average between 50 °C to 80 °C, and in some cases modified starches exceed this and gelatinise at far higher temperatures. It is dependent on amylopectin cross linkages and amylose/amylopectin ratios. Decreased amylose content equates to an increase in gelatinisation temperature. According to Belitz *et al.* (2004) potato starch has an amylose content of approximately 23% and a gelatinsation temp between 58-66 °C. Waxy corn (commonly known as maize) starch has between 0-1% amylose content and a gelatinisation temp of 63-72 °C [24].

Enzyme activity will be measured using gelatinised starch solutions with the addition of α-amylase or diastase (α and β amylase derived from malt) to catalyse the breakdown of the starch. Measurements will be taken to determine the ultrasound velocity as the starch solution is metabolised by the enzyme.

Using this approach, it is possible to monitor the reaction between enzyme and substrate through change in speed of sound. This is an efficient, *real time* process allowing the enzymic activity to be observed rather than just the initial rate. The measurements reported here improve

on work first carried out in 1983 by Povey and Rosenthal whom investigated the ultrasonic detection of the degradation of Starch by α-amylase [25].

In the following sections we shall describe the use of ultrasound to monitor enzyme activity upon low concentration gelatinised starch gel samples. To corroborate findings ultrasound measurements will be taken using two devices, the Cygnus UVM ultrasound velocity meter (Cygnus Instruments, Dorchester, U.K) and the Resoscan TF Instruments. Additionally, rheological measurements (Kinexus Rheometer, Malvern, UK) of the gels pre and post enzyme to illustrate the changes in gel network properties and optical microscopy (Celestron LCD Digital microscope Model No. 44340) to show starch cell breakdown.

2. Materials and methods

2.1. Sample preparation

Starch solutions of 3% w/v and 5% w/v were prepared by the addition of native potato starch (NPS) or maize starch (at either 3 g or 5 g) to 100 ml millipore water (0.22 μm filter) and heated at 70 °C for 30 minutes to to ensure the starch is gelatinised and all granules are fully hydrated. Samples were continuously stirred with a magnetic flea to ensure uniform heating and samples were thoroughly mixed. The presence of dehydrated starch granules will disrupt attenuation and cause inaccuracies. The sample is then cooled to the required temperature (typically 37 °C) for analysis to take place.

A 1% w/v enzyme solution was prepared by either 100 mg α-amylase or diastase in 10ml millipore water and heated to 37°C to ensure the enzyme was fully hydrated but not inactivated. α-amylase (MP Biomedicals, LLC) origin from Bacillus subtilis, containing approximately 165 bacterial Amylase units per mg. One unit will dextrinize 1 mg of starch per minute at pH 6.6 and 30°C. Diastase (α and β amylase) (Fisher Scientific, UK) was derived from malt.

2.2. Ultrasound measurements

Ultrasonic velocity measurements were taken using a Cygnus UVM ultrasound velocity meter (Cygnus Instruments, Dorchester, U.K) schematic shown in figure 1 and, for comparison using a Resoscan instrument (TF Instruments, Heidelberg, Germany [26]) see figure 2.

2.2.1. Cygnus UVM. The UVM comprises of a cylindrical steel vessel (interior diameter 40 mm, capacity 75 ml) equipped with a 2MHz ultrasonic transducer which emits pressure wave pulses which travel through the sample medium hits the cell wall and returns to the transducer where the time of flight and the temperature will be recorded simultaneously. The cell is located within temperature controlled tank with 0.1°C uniformity (Grant Instruments, model GE15). The sample was stirred continuously using a magnetic flea to ensure a consistent temperature throughout the sample. This aspect is important since ultrasound velocity is temperature dependent. For example, in water at 25 °C every 0.1 °C change in water equates to a 0.3m s^{-1} change in velocity [13]. The UVM is initialised using a certified referenced temperature probe (Fluke, CHUB-E4 1529) and associated reference velocity. The UVM takes measurements 10 times a second and records the average and standard deviation (coefficient of variation less than 0.01%). This process allows changes in state of the medium to be correlated with ultrasound velocity measurements.

Gelatinised starch solutions of 3% w/v or 5% w/v were examined by placing 100 ml into the UVM cell with the addition of 1 ml of 1% w/v enzyme aliqout (10 mg enzyme). Upon the addition of either α-amylase, β-amylase or diastase into the cell, catalysis of the polysaccarides in the viscous soultions cause a reduction in viscosity equating to an increase in the velocity of sound.

2.2.2. Velocity reading validation. To establish that the observed differences in velocity were not merely the addition of the enzyme addition, an aliqout of 1 ml of 1% w/v enzyme solution was pipetted into the UVM cell containing only millipore water at 37 °C and provided an approximate

velocity of 1523.65 ± 0.1 ms^{-1}. After addition of the enzyme solution the recorded velocity was approximately 1523.65 ± 0.1 ms^{-1}. Since the UVM supplies average estimates, a T-test was applied with no statistically significant changes observed between pre and post-addition of the enzyme aliquot (p-value > 0.05).

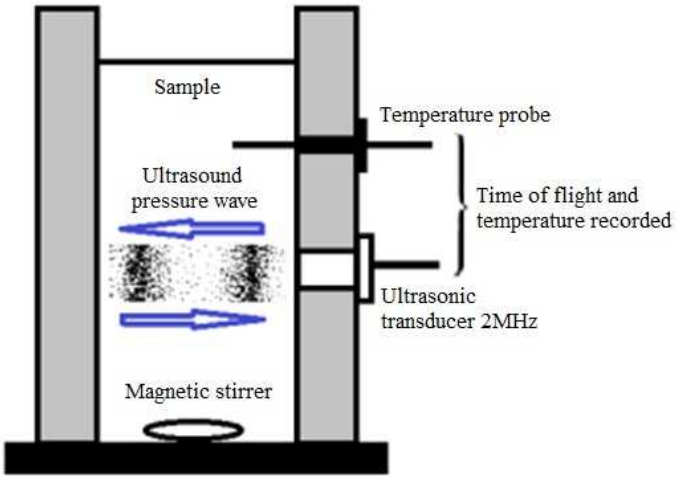

Figure 1. Schematic representation of the Cygnus UVM cell (Cygnus Instruments, Dorchester, U.K).

2.2.3.

2.2.4. Resoscan. Further ultrasound velocity measurements of gelatinised 3% w/v NPS solution were collected using a Resoscan, TF Instruments. This is a high precision relative measurement device (repeatability 0.01 ms^{-1}) which simultaneously collects data from two sample cells determining both the the ultrasonic velocity and attenuation of ultrasound pulses within the samples. Cells have a capacity of 200 µl, and the operational frequency range of 7.3-8.4 MHz, with the temperature controlled (±0.05 °C) by a Peltier thermostat. Data was automatically gathered by the instrument providing ultrasound velocity readings for starch solutions pre and post-enzyme addition.

Initially both cells contained 200 µl millipore water to verify accuracy against reported literature sources [13, 14]. Readings from both cells were consistent with one another. 100 ml gelatinised 3% w/v NPS solution was prepared, 200 µl was pipetted into each cell and readings were collected to ensure both cells were consistent with one another. The cells are flushed with millipore water and heated to 70 °C to remove residues before placing further samples into the cells.

Another batch of 100ml gelatinised 3% w/v NPS solution was prepared and kept on a heated plate at 37 °C containing a magnetic flea, 200 µl is pipetted into the first cell. Subsequently an aliqout of 1ml of 1% enzyme solution is pipetted into 100ml NPS sample which is stirred by the magnetic flea, then 200 µl is pipetted into the second cell.

It must be noted that there was an approximate 5 second delay between the addition of the enzyme to the solution and the placing of solution into the Resoscan cell.

2.3. Rheological measurements

Visco-elastic properties of the starch gels pre and post enzyme metabolised was conducted using a temperature-controlled Kinexus rheometer (Malvern, UK) using cone-plate arrangement (60 mm diameter cone with 2^o angle and a 65 mm diameter plate). Approximately 2 ml of sample is applied to the plate at temperature is then changed to the required value 25 ^oC and, after 30 minutes of aging, measurements are performed. Oscillatory (0.1 – 10 Hz) rotational strains were applied to the sample, generating a vibratory stress to supply the complex shear moduli in the pre-enzyme gels as described

in equation (3). Strain sweeps with shear rates 1-300 s^{-1} were implemented to generate apparent viscosities η (Pa s).

Figure 2. Resoscan instrument by TF Instruments, Heidelberg, Germany [26].

2.4 Optical microscopy

For qualitative analysis, optical microscopy was employed enabling still and video images of reactions between enzyme and substrate to be taken. All images have a 40X magnification. A Celestron LCD Digital microscope Model No. 44340 was used to visualise the time course of enzyme reaction taking place. Gelatinised starch samples of Native potato starch and maize starches were used with small quantities of enzyme solution were carefully pipetted onto the glass slide. Video sequences were captured enabling real time reactions to be visualised of the gelatinised samples before and after addition of the enzyme solution at increasing times upto 5 minutes.

3. Results and discussion

In the following sections various experimental results relating to ultrasonic detection of enzyme addition, velocity dependence upon temperature, starch sample concentration, rheology and optical microscopy will be presented.

3.1. Addition of enzyme in pure water and ungelatinsied samples.

Due to the sensitivity of ultrasonic techniques it was important to identify if the addition of the enzyme solution into the starch sample during analysis produced any significant changes in the speed of sound to verify that the observed change in ultrasonic velocity was not an simply an artifact of the addition of the enzyme solution or change in temperature. An aliqout of 1 ml of 1% w/v enzyme solution was pipetted into the UVM cell containing only millipore water at 37 °C and provided an approximate velocity of 1523.65 ± 0.1 ms^{-1}. After addition of the enzyme solution the recorded velocity was approximately 1523.65 ± 0.1 ms^{-1}. Since the UVM supplies average estimates, a T-test was applied with no statistically significant changes observed between pre and post-addition of the enzyme aliquot (p-value > 0.05).

This process was also applied to starch samples that had not been fully gelatinised (i.e. heated to temperatures < 50 °C, see figure 6 (a) and (e)) rendering the polysaccharides unvailable for metabolsim by the enzyme. No difference in ultrasound velocity was similarly noted.

3.2. Variation of ultrasound velocity measurements in relation to sample concentration.

Ultrasound measurements were collated from the UVM to examine how the concentration of the sample affects the speed of sound. Figure 3 shows 3% and 5% w/v gelatinised NPS solutions at 37°C. Velocity determinations were conducted using replicates. The data plotted in figure 3. Show running averages over 20 samples. As reported the UVM records average values based upon 10 measurements and also records the associated standard error (normally < 0.2 m s^{-1}) of the measurements. Note in Figure 3 that 3%w/v data have been translated by 4.5 ms^{-1} from 1526.5 ms^{-1} in order to show the overlay over the 5% w/v velocity data. α-amylase is added at approximately 200 seconds and subsequent hydrolysis with α-amylase of 3% and 5% w/v samples can be noted

with an associated change in the ultrasound velocity. Enzyme activity rate can be seen to differ between the different concentrations. Enzyme action affects the speed of sound and the substrate concentration the difference in the final velocity achieved.

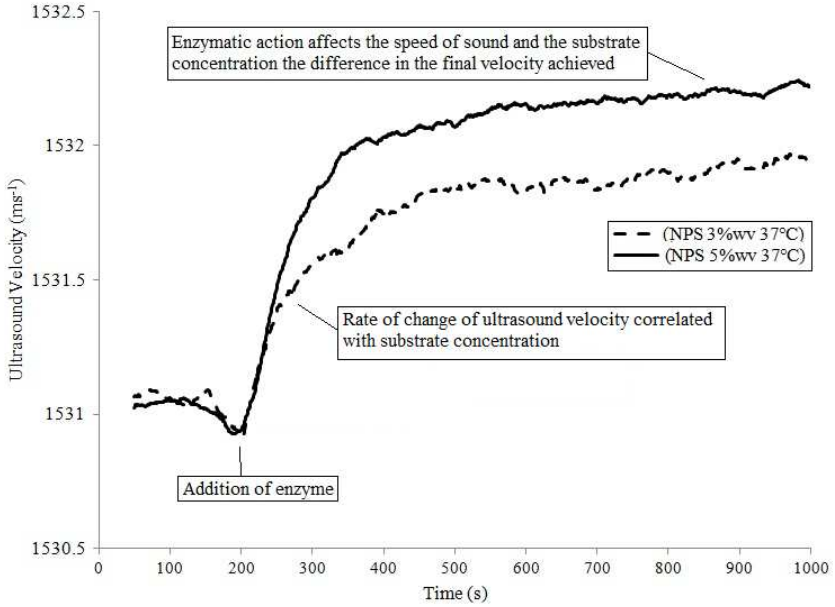

Figure 3. Ultrasound velocity (ms^{-1}) measurements against time (s) using the UVM for gelatinised Native Potato Starch (NPS) and water at 37°C; 5%w/v (solid line) and 3%w/v (dashed line)

The coefficient of variation is less that 0.01% therefore no errors bars are necessary on the plotted data. The initial velocities in the 3% w/v gel is 1526.6 ms^{-1} and in the 5% w/v gel 1531.0 ms^{-1} therefore the 3% w/v data has been translated by 4.5 ms^{-1} to enable comparison with the 5% w/v results. As can be seen, there is a increase in ultrasound velocity in both samples after the addition of the 1% enzyme solution at approximately 200 seconds (statistically significant p-values<0.001). Specifically, there is a greater absolute change in the velocity and in the rate also between the different concentrations. This demonstrates that ultrasound is able to detect enzyme rate activity over different concentration of the substrate and furthermore provides a real time observation of the kinetics thereby potentially removing the need for additional experimental analyses as discussed in the the introduction. The addtion of α-amylase results in the catalysis of starch from long chains, into short chained dextrins. This process in know as dextrinisation and can be seen in figure 3 to continue over approximately 6 minutes.

To ensure that the enzyme reaction had indeed metabolised all the substrate and that the enzyne itself had not been deactivated an additional aliquot of α-amylase solution was added to meatbolised sample. A further innoculation of 10mg α-amlyase showed no further change in velocity readings confirming that readings taken from the UVM relate to the reaction between enzyme and substrate itself rather than the addition of enzyme solution. This process was repeated with consistent findings.

3.3. Variation of ultrasound velocity measurements with temperature

Ultrasound measurements were collected using the UVM demonstrating changes in ultrasonic velocity for pre and post α-amylase addition in gelatinised 5% w/v NPS samples over several temperatures 26, 29 and 37 °C.

Figure 4 demonstrates that an increase in the speed of sound within the samples as the

temperature is increased, consistent with an increase arising from the temperature dependent speed of sound in the water component of the starch gel. Furthermore the figure identifies an increase in ultrasonic velocity in post α-amylase addition. Post enzyme velocities reported are those when the velocity reaches a stable asymptotic value as noted in the inset graph.

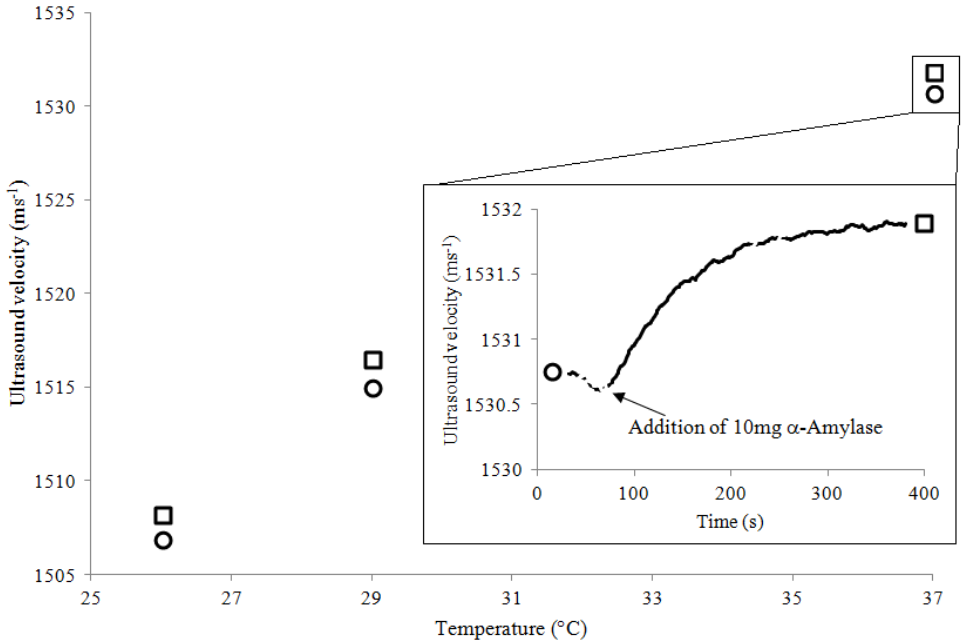

Figure 4. Speed of sound (ms^{-1}) in 5% w/v gelatinised Native Potato Starch (NPS) and water against temperature (°C). Circles show velocities prior to introduction of enzyme and squares after hydrolysis with α-amylase. Inset graph shows speed (ms^{-1}) against time (s) at 37 °C. The UVM performs ten velocity readings every second and the average with standard error (normally < 0.2 m s^{-1}) is recorded. Coefficient of variation is less than 0.01% so no error bars are shown.

3.4 Rescoscan measurements

Values taken from the UVM and Resoscan were compared to ensure similar relative pre and post enzyme velocity measurements were recorded. Table 1 shows speed of sound readings taken from both the UVM and the Resocan, giving equivalent readings for both pre and post α-amylase addition. This suggests that there is a significant level of repeatability when using these techniques.

Table 1. A table showing comparisons of measured ultrasound velocity ν (ms^{-1}) of NPS 3%w/v using the Resoscan and the UVM.

Instrument	Temperature/ precision (°C)	Velocity precision (ms^{-1})	Velocity (ms^{-1})	
			Pre α-amylase	Post α- amylase
Resoscan[a]	37.0 ± 0.05	± 0.01	1526.7	1527.8
UVM[b]	37.0 ± 0.1	± 0.1	1526.7	1527.8

[a] Simultaneous velocity and attenuation taken in the frequency range 7.3-8.4 MHz.

[b] UVM frequency 2MHz, ten velocity readings taken every second and the average with standard deviation recorded. Coefficient of variation less than 0.01%.

Although the Resoscan is a high precision device it is more problematic in measuring velocity and attenuation in the gelatinised starch samples. This is primarily due to the small sample sizes required which makes it difficult to prepare the samples and also to monitor the evolution of the sample since premixing is required before presentation to the sample cells.

3.5 Rheology measurements

Rheological measurements of the 3% and 5% w/v gelatinised NPS starch gels pre and post enzyme metabolised were conducted using a temperature-controlled Kinexus rheometer (Malvern,UK) using cone-plate arrangement. Figure 5 shows the results obtained from the frequency sweep tests which show a reduction in apparent viscosity of 3% and 5% samples as shear rate increases. Furthermore, it identifies significant reduction of viscosity in samples post enzyme showing approximately 4-5 orders of magnitude reduction in the apparent viscosity η (Pa s). Measured stress measurements varied between 20-600 Pa in the gel phase and between 10^{-3} and 1.5 Pa in the post enzyme more liquid phase solutions. The oscillation tests produce values of around 10^2 Pa storage modulus G' measurements and order 10 Pa for the loss modulus G'' in the gel phase. Reductions in the viscosity and storage modulus evidently impacting upon the longitudinal velocity measurement as indicated in equation (6). In Figure 5 enzyme activity reduces the viscosity by orders of magnitude resulting with in a small increase in ultrasonic velocity within the medium. Within the gels, the storage modulus G' is of the order 10^2 Pa and loss modulus G'' is of the order 10 Pa. Rheology measurements were performed using the Kinexus rheometer (Malvern,UK)

3.6 Optical Microscopy

Qualitative results of the enzyme action upon the starch gels is shown in Figure 6 in which images taken from video sequences are generated by observing NPS samples before complete gelatinsisation after gelatinisation and then the sequence after the addition of the enzyme solution which progressively breaks down the starch cells, specifically by dextrinisation of the polysaccharides. Figure 6 **(**a) shows the ungelatinised NPS solution heated to approximately ~ 50 °C therefore complete gelatinsation was not achieved. Note cell striations characteristic of partially swollen starch granules. Figure 6 (b) shows the initial gelatinised starch gel (as described in the introduction) at the point where α-amylase added to commence catalysis. Note several starch granules still remain. Figure 6 (c) α-amylase progressively breaks down the starch cells over several minutes continually reducing viscosity. Figure 6 (d) Gelatinised cells broken down completely, starch granules remain. Figure 6 (e) ungelatinised maize starch (MS) cells heated ~ 50 °C. Note smaller cell size. Figure 6 (f) Diastase α–β amylase added to initial gelatinised sample. Figure 6 (g) Diastase breaks down starch cells. Figure 6 (h) All starch cells will finally be broken down. All images 40X magnification using a Celestron LCD Digital microscope Model No. 44340.

4. Conclusion

We have investigated the use of ultrasound to monitor enzyme activity in gelatinised starch samples. Various concentration and temperature conditions were examined. The results indicate that the rate of enzyme reaction of different concentrations of enzyme or substrate can be measured by ultrasound velocity. The technique permits time dependent measurement over a wide temperature range. Changes in ultrasound velocity in pre-dextrinised gels and subsequently dextrinised were monitored dynamically and related to concentration levels of the substrate (polysaccharide) remaining. Differences in velocity are also evident between different concentrations of the starch samples and at different temperatures. Findings are corroborated with optical microscopy and rheological measurements. Ultrasound utilises acoustic pressure waves to probe the material properties of the sample medium and can be performed without the need for aliquot extraction; this offers an efficient, non-invasive, non-destructive and dynamic method to monitor enzyme activity in potentially a wide

range of applications.

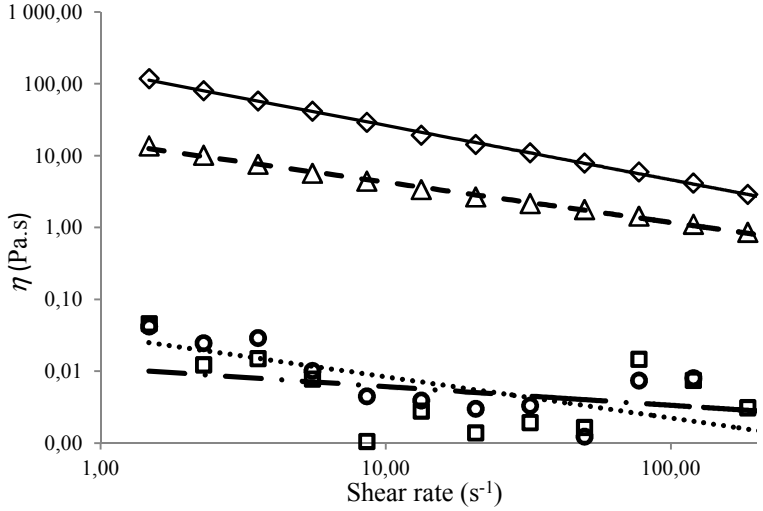

Figure 5. Apparent viscosity η (Pa s) against shear rate (s^{-1}) of gelatinised Native Potato Starch (NPS) and water at; 5% w/v (solid line with diamonds), 3% w/v (dashed line with triangles), 5% w/v after hydrolysis with α-amylase (dashed-dotted line with square) and 3% w/v after hydrolysis with α-amylase (dotted line with circles).

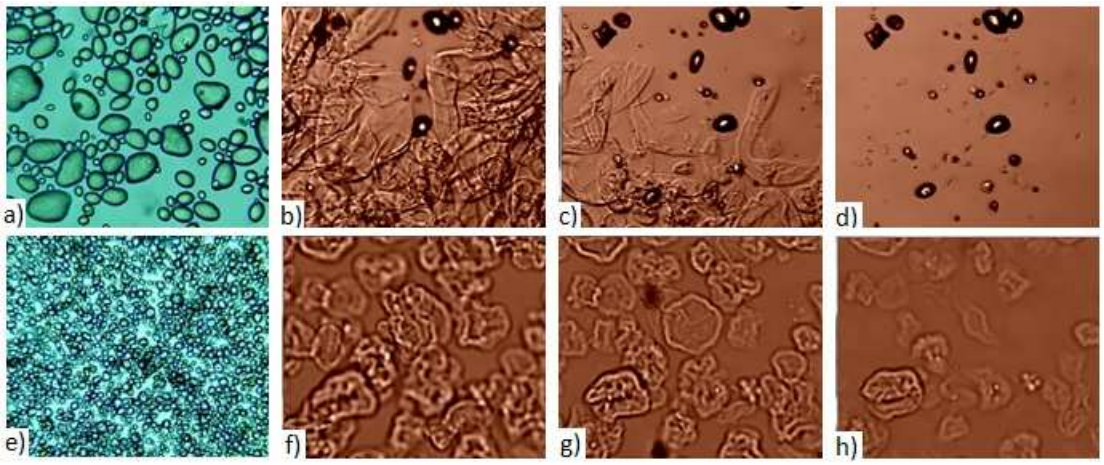

Figure 6. a) Ungelatinised NPS heated ~ 50 °C. Note cell striations characteristic of partially swollen starch granules. **b)** α-amylase added to gelatinised starch cells. Note starch granules. **c)** α-amylase breaks down starch cells over several minutes. **d)** Gelatinised cells broken down entirely, starch granules remain. **e)** Ungelatinised maize starch (MS). cells heated ~ 50 °C. Note smaller cell size. **f)** (Diastase) added α–β amylase. **g)** α–β amylase (Diastase) breaks down starch cells. **h)** All starch cells will finally be broken down. All images 40X magnification using a Celestron LCD Digital microscope Model No. 44340.

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