SASTA OLIPHANT SCIENCE AWARDS

SOUTH AUSTRALIAN SCIENCE TEACHERS ASSOCIATION

Highly Commended

Scientific Inquiry

Year 11-12

Asira Suetrong

Glenunga International High School

Investigating The Effect of Porcine Trypsin Concentration on The Rate of Hydrolysis of Casein in Skim Milk

Research Question

How do varying concentrations of porcine trypsin (1%, 2%, 3%, 4%, 5%) affect enzymatic activity in the rate of hydrolysis of casein in skim cow's milk, as measured by the change in absorbance after 5 minutes (AU/min) using colourimetry.

Introduction

Our living functions are facilitated by biochemical processes as they are catalysed by enzymes to ensure a sufficient output capable of sustaining life. As methodologies used in surgical and traumatic recovery have improved over time, the case of inflammation is still inherent as it is an innate response in the healing process. While inflammation indicates the flow of leucocytes and thus, an immune response in preventing infections, it is often uncomfortable and in severe cases, can stimulate necrosis in tissue and multisystem organs (Margraf et. al. 2020). Trypsin is a proteolytic enzyme that can be applied in pharmaceutical therapies as it catalyses the hydrolysis of peptide bonds found in proteins, aiding in their absorption and in blood flow. As an aspiring biochemical researcher, I wanted to apply my knowledge of enzymes and further explore their functions, particularly in trypsin and how it can improve postoperative recovery and trauma. This is especially important when determining the most effective enzyme concentration to ensure efficient anti-inflammatory action whilst minimising the costs of therapy.

To investigate the effect of trypsin concentration on enzymatic activity, concentrations of 1%, 2%, 3%, 4%, and 5% will be tested on skim milk as the source of the substrate; casein is a white-coloured quaternary protein found in dairy products and when hydrolysed, the smaller molecules become soluble and subsequently, turn the milk clear. Skim milk will be used to ensure that the opacity of the solution is minimally obscured by the naturally-present triglycerides in full-cream milk. As a result, the rate of hydrolysis can be measured in the change of opacity. While this change may be measured by eye, I will be using colourimetry to quantify the change in absorbance of the solution over 5 minutes. This will ensure objective measurements while still considering qualitative observations.

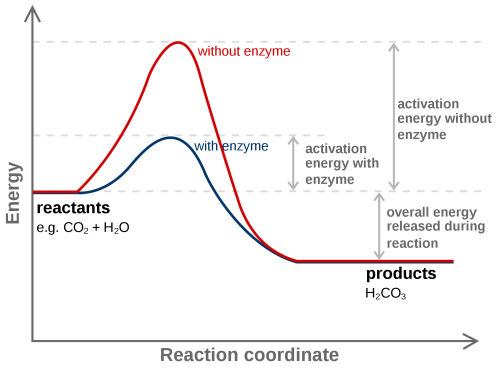

Figure 1: Energy Profile Diagram of a Catalysed Exothermic Reaction (Kilbee 2014)

Background Information

Trypsin is a globular serine protease that catalyses the hydrolysis of peptide bonds by cleaving peptides at the C-terminal side of the amino acid. This catabolic process in casein separates the protein molecules into dipeptides, tripeptides, and free amino acids. As the monomer of proteins, amino acids are composed of an amine and carboxyl group, bonded to the central carbon.

Casein, also a globular protein, is naturally found in dairy products, making up around 80% of milk proteins (Pikosky 2016). Pure casein is white when suspended in milk and when consumed, is digested by enzymes secreted by the pancreas that hydrolyse its peptide bonds, breaking the molecule down into more soluble amino acids. As a result, the amino acids become a colourless solution.

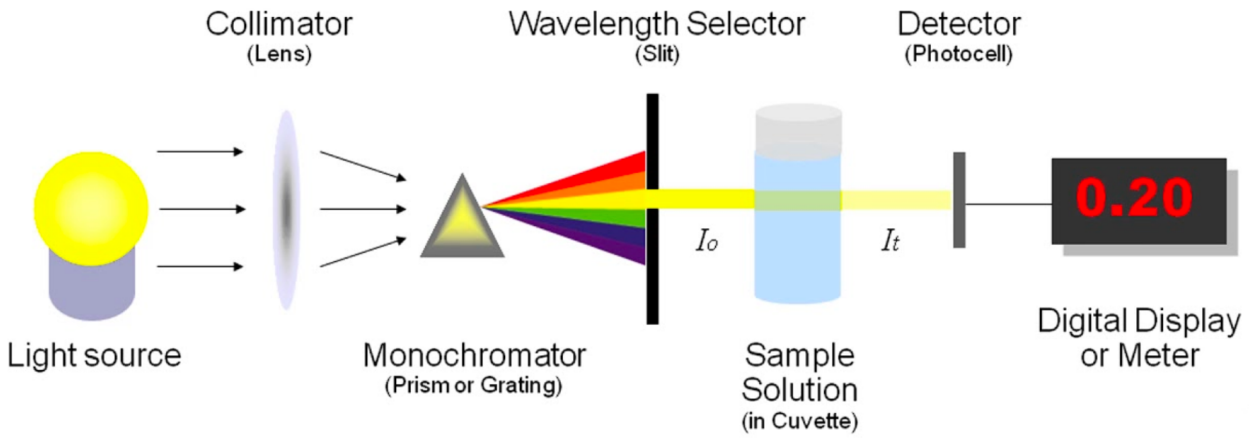

Figure 2: The Primary Components of a Colourimeter (TOPPR 2023)

By applying Beer-Lambert's Law, which states that there is a direct relationship between the absorption of light transmitted through a medium and its concentration (Arnold 2015), the rate of casein hydrolysis can be determined using colourimetry. A colourimeter measures absorbance (AU) by passing a beam of light with a specific wavelength through a solution. This light enters a collimator before passing through the solution in order to narrow the coloured light into the measuring device (see Fig. 2). Theoretically, as the milk solution should turn more translucent with the addition of trypsin, there should be a lower concentration of casein and thus, fewer substances that absorb light. As the device measures the amount of light absorbed by the substance, its transmitted light must be the same. This assumption helps inform the use of a wavelength of 635nm as this is a red-orange colour; skim milk has a bluish tint due to the scarcity of fat globules, giving rise to the Tyndall effect (Hewitt 2023; Helmenstine 2019), and so its opposite colour (red) is absorbed (see Fig. 3). Colourimetry measures the colour change from the hydrolysis of casein in milk, providing this experiment with both qualitative and quantitative data.

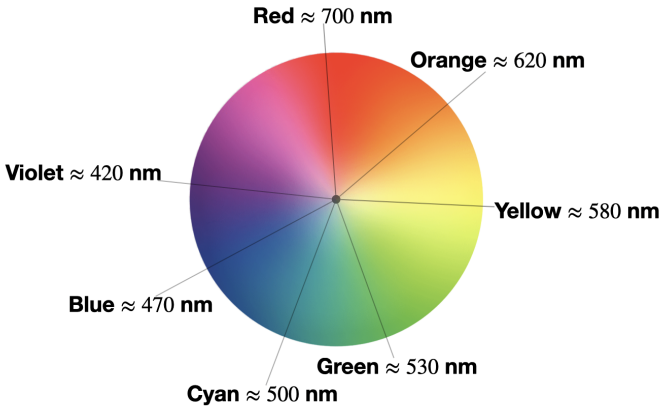

Figure 3: Colour wheel with approximate wavelengths of colours (Haas 2020)

Aim

The aim of this experiment is to investigate the effect of varying porcine trypsin concentrations (1%, 2%, 3%, 4%, 5%) on the rate of hydrolysis of casein as found in skim cow's milk, and as measured by the change of absorbance (AU/min)

Hypothesis

It is predicted that as trypsin concentration increases, the rate of casein hydrolysis will also increase, as there are more enzyme molecules to increase the frequency of collisions and potential binding to substrates (Cornell 2016). However, enzymatic activity will ultimately plateau as all enzyme molecules are completely saturated with casein, its substrate (Robinson 2015; Liu 2017). Therefore, it is hypothesised that 5% trypsin will result in the highest rate of casein hydrolysis, with 1% trypsin causing the lowest.

Variables

Independent variable:

The concentration of porcine trypsin (1%, 2%, 3%, 4%, 5%) that is used for each trial. This range encompasses the concentrations used in a variety of trypsin therapies, including as an oral anti-inflammatory drug, fibrinolytic drug, and cell dissociation (Sigma-Aldrich 2023). However, 0% trypsin was not used as a controlled trial as the hydrolysis of casein is significantly prolonged in the absence of enzymes; if a human body does not produce trypsin, this can lead to malabsorption as complex proteins such as casein are not broken down at a sufficient rate (Sethi 2018).

Dependent variable:

The rate of casein hydrolysis after 5 minutes, as measured by the change of the solution's absorbance (AU/min).

Table 1: Controlled Variables

Controlled Variable	Method of Control	Effect on the Experiment
Volume of trypsin	All trials will use 1ml of trypsin at its respective concentration, as measured using a 2ml micropipette.	The volume of trypsin must be controlled as varying the the volume of the enzyme would lead to different rates of reaction; this would introduce another independent variable and hinder the correlation between enzyme concentration and rate of hydrolysis.
Type of trypsin	All trials will use porcine trypsin.	Trypsin with varying derivatives may function slightly differently and hence, produce varying results due to their differences rather than being caused by enzymatic concentration. (Johnson & Marshall 2002)
Type and fat concentration of milk	All trials will use Coles Australian skim cow's milk from the same batch (<0.1% fat).	Milk from varying origins (ie. cow and goat) may differ in casein concentration and other extraneous factors. Skim milk is chosen as the triglycerides/lipids found in milk can be a slight yellow hue due to carotenoids. If there are varying concentrations of lipids, the opacity of the solution will differ and thus, produce unreliable data (Hewitt 2023; Helmenstine 2019).
Volume of milk	All trials will use 2ml of skim cow's milk, as measured using a 2ml micropipette	As the source of casein, which is acting as the substrate for trypsin, the volume of milk must also be controlled to ensure that all trials are using the same amount of substrate. More of the substrate may potentially engender more hydrolysis to occur simultaneously.
Wavelength of colourimeter	A colourimeter set at 635nm will be used	The wavelength of the colourimeter must be controlled as it measures the substance's absorption of the light being

	throughout all trials.	transmitted by the device in order to determine absorbance. At this same wavelength, a slight change in the concentration of casein in milk can lead to a significant response in the absorption of light. As a result, it is important that the wavelength is controlled to isolate the correlation between trypsin concentration and the rate of casein hydrolysis.
Duration of each trial	Each trial will last 5 minutes as a recording is taken at every 30-second increment. These recordings will be made on Vernier Graphical Analysis, which constructs a continuous line graph and from that, the data can be recorded.	As the dependent variable is the rate of casein hydrolysis, varying lengths of time allotted to each trial will lead to different results consequenced by an extraneous factor. A trial that is allowed more time than another will have the opportunity for more enzymatic activity to occur and hence, will give a higher rate of reaction that cannot be used to compare data for correlation.
Solvent used in trypsin solutions	All trypsin solutions will use 10g of distilled water with either 0.1g, 0.2g, 0.3g, 0.4g, or 0.5g of powdered trypsin.	Distilled water is free of any contaminants of minerals or dissolved ions and has a neutral pH of 7. Varying pH can affect the enzyme due to the isoelectric points of its amino acids; in a solution with a low pH, there is an abundance of H^+ ions, changing the charge of the amino acid into a cation and conversely, OH^- in alkaline solutions may cause the amino acid to turn into an anion (Davis 2021). As a result, using distilled water as a solvent will not impact the enzyme function.

Uncontrolled Variables:

- Temperature change: due to the nature of the colourimeter, the experiment's temperature was not able to be controlled with a water bath or on a block heater. However, as none of the materials requires refrigeration and can remain within the room throughout the entire experiment, temperature changes will be concurrent with all of the collected data.
- Natural degradation of trypsin (cumulative deterioration): as a globular protein, trypsin is sensitive to changes in temperature, pH, and time as oxidation, racemization, and other chemical processes alter parts of the protein molecule. Additionally, trypsin is highly active and can exert its catalytic function on itself (Scripps 2023). This natural degradation cannot be controlled although completing all trials within a short period of time will minimise the impact of this extraneous factor. The enzyme will also be stored at room temperature (~21℃) which is within trypsin's zone of tolerance. Temperatures above 65℃ will have too much kinetic energy and denature the enzyme by disrupting its intramolecular forces while temperatures lower than 20℃ will make trypsin inactive as there is not enough energy.
- Enzymatic activity that occurs when trypsin is ejected from the micropipette and the colourimeter lid is replaced: as the enzyme cannot be added to the milk simultaneously to the lid's closure, there will be some enzymatic activity that is not recorded nor accounted for. The impact of this variable will be alleviated by starting the data collection before ejecting the enzyme and replacing the lid immediately. As Graphical Analysis plots the data on a continuous line graph, I will be able to choose the starting time to the nearest second and record the 30-second intervals following.
- Potential impurities in trypsin and milk: however, as the same sources of trypsin and milk are used in all trials, this would potentially pose a systematic error.

Apparatus:

- 25 x 4.5ml Cuvettes
- 50ml Coles Australian Skim Milk
- 1.5g Porcine Trypsin
- 50ml Distilled Water (for trypsin solutions)
- 2 x 2ml Corning Micropipette (±0.005ml)
- 26 x Micropipette tips
- 5 x 50ml Conical Tubes (only used to hold solutions; all volumetric measurements are made using a micropipette)
- Electronic scale (OHaus Pioneer Analytical Balance) (±0.001g)
- Vernier Colourimeter (±0.001 AU), connected to laptop with USB cable
- LabQuest Mini Interface
- Distilled water (for washing)
- Glass Stirring Rod
- Black background (ie. card/paper)
- 1 x Spatula

Procedure:

Part A: Preparing Trypsin Solutions

1. Wash conical tubes and glass stirring rod with distilled water and let dry.
2. Measure 0.1g of powdered trypsin using the Serrata electronic scale into a conical tube.
3. Into the same conical tube, measure 10g of distilled water using the scale. As 10g of distilled water is equivalent to 10ml of distilled water, measuring the solution by mass removes the influence of any parallax error that would be present if it is measured by volume. Making these measurements directly into the conical tubes also removes the need for transferring contents and overall, improves the precision of these measurements.
4. Stir the solution with a glass stirring rod until the solution is fully dissolved.
5. Repeat steps 1-4 with the remaining concentrations of 2%, 3%, 4%, and 5%, with 0.2g, 0.3g, 0.4g, and 0.5g, respectively.

Part B: Calibrating the colourimeter

1. Set the wavelength of the colourimeter to 635nm.
2. Fill a cuvette with distilled water and place into the device's cavity, ensuring that the arrow is facing inwards.
3. Press the 'CAL' button and allow the red light to cease flashing.
4. Remove the cuvette.

Part C: Experiment

1. Connect the colourimeter to the laptop using a USB cable and a Labquest Mini Interface.
2. In a cuvette, measure 2ml of milk using a 2ml micropipette and place into the device's cavity.
3. Measure 1ml of 1% trypsin using a micropipette set aside.
4. Start the data collection on Graphical Analysis and eject the trypsin solution into the cuvette, replacing the device cover immediately.
5. Allow data collection to occur for 5 minutes, as determined by Graphical Analysis.
6. Remove cuvette and place it in front of a black background to record qualitative observations.
7. Repeat steps 3-6 another 4 times for 5 trials in total.
8. Repeat step 7 with the remaining trypsin concentrations (2%, 3%, 4%, 5%).

Safety, Environment, and Ethical Considerations:

Table 2: Risk Assessment

Risk	Hazard Statement	Precautionary Statement	Treatment
Plastic conical tube	Plastic is a porous material and even when washed liberally, may contain residual traces of previously held substances. Some organic solvents may also affect the plastic, causing leaks.	All conical tubes (one per trypsin concentration) will be new and washed with distilled water.	If leaking occurs, discard the tube and clean up the workspace. As the tube is only meant to contain trypsin and water in this experiment, this can be washed in the sink.
Trypsin (powder)	The fine dust-like nature of powdered trypsin poses the risks of inhalation, ingestion, and the substance entering the eye.	Wear safety goggles and ensure that the powder is not handled in close proximity to the face.	In the event of ingestion, drink water to lower its concentration and seek medical attention. Wash eyes immediately if trypsin makes contact with them.
Colourimeter	As an electronic device that must be connected to a laptop, contact with water may cause faults and accidents. Dropping the device may also lead to malfunction.	Ensure that the device does not make contact with water and that inserting the solution-filled cuvette is being done so with care as to prevent spillage. Care should also be taken to avoid any collisions or impacts with a hard surface.	If fire erupts, extinguish it immediately and seek professional aid. If the device is broken upon impact, clear the area and ensure that no broken pieces remain.
Glass stirring rod	Glass will shatter upon impact and shards may cause cuts or abrasion.	Ensure that the cylindrical rod is placed on a secure surface to prevent rolling.	If cut, wash the site with warm, soapy water, and remove any shards with sterilised tweezers. Apply pressure to stop bleeding and seek medical attention if necessary.

Ethical Considerations:

- As the trypsin is of porcine origin, is it important to acknowledge its animal-derived origins. While there are some fungal proteases available, trypsin can only be obtained by extraction from frozen pancreatic glands. Milk is also an animal-derived product, however, as the intended substrate for this experiment is casein, there can only be mammalian dairy products; plant-alternatives do not contain casein.
    - Both of these materials will be used in very small quantities (1.5g trypsin power and 50ml milk) as to prevent wastage and ethical impact.

Environmental Considerations:

- All materials employed in this experiment have no or very low toxicity and can be disposed of down the laboratory sinks. Subsequently, there are no significant environmental risks present in this experiment.

Raw Data

Absorbance is collected using a Vernier Colourimeter while connected to a laptop with LabQuest Mini Interface. The data is taken from a continuous line graph of time (±1 second) against absorbance (±0.001 AU).

Table 3: Absorbance of Milk After Every 30 Seconds For 5 Minutes With 1%, 2%, 3%, 4%, 5% Trypsin

Trypsin Concentration (±0.2%)	Trial	Absorbance (±0.001 Au) of Milk Over 5 Minutes										
		0s	30s	60s	90s	120s	150s	180s	210s	240s	270s	300s
1	1	0.623	0.538	0.468	0.397	0.347	0.310	0.291	0.269	0.237	0.213	0.194
	2	0.650	0.546	0.436	0.389	0.333	0.302	0.266	0.250	0.238	0.219	0.197
	3	0.572	0.474	0.347	0.302	0.233	0.221	0.205	0.196	0.193	0.176	0.163
	4	0.521	0.454	0.347	0.286	0.260	0.239	0.224	0.208	0.189	0.177	0.166
	5	0.555	0.520	0.444	0.376	0.329	0.279	0.256	0.241	0.221	0.208	0.190
2	1	0.629	0.566	0.512	0.441	0.391	0.350	0.308	0.282	0.256	0.233	0.219
	2	0.657	0.618	0.526	0.453	0.401	0.356	0.310	0.282	0.252	0.232	0.217
	3	0.711	0.647	0.560	0.495	0.439	0.383	0.338	0.305	0.276	0.248	0.225
	4	0.558	0.568	0.484	0.424	0.370	0.330	0.297	0.269	0.249	0.229	0.214
	5	0.662	0.576	0.512	0.454	0.411	0.362	0.326	0.295	0.264	0.243	0.220
3	1	0.669	0.588	0.425	0.291	0.220	0.194	0.193	0.182	0.180	0.169	0.157
	2	0.581	0.496	0.322	0.205	0.151	0.130	0.122	0.116	0.115	0.118	0.116
	3	0.663	0.494	0.351	0.207	0.175	0.170	0.162	0.161	0.145	0.130	0.126
	4	0.579	0.488	0.396	0.258	0.209	0.182	0.165	0.153	0.150	0.147	0.144
	5	0.641	0.487	0.378	0.258	0.239	0.205	0.207	0.198	0.189	0.182	0.166
4	1	0.551	0.409	0.251	0.153	0.051	0.032	0.022	0.017	0.016	0.015	0.015
	2	0.490	0.409	0.282	0.153	0.053	0.039	0.037	0.033	0.028	0.026	0.022
	3	0.557	0.446	0.315	0.190	0.101	0.062	0.046	0.042	0.042	0.039	0.040
	4	0.537	0.413	0.342	0.197	0.092	0.024	0.019	0.017	0.016	0.015	0.011
	5	0.550	0.419	0.312	0.180	0.087	0.044	0.037	0.022	0.018	0.017	0.017
5	1	0.508	0.366	0.161	0.095	0.023	0.018	0.014	0.013	0.011	0.011	0.011
	2	0.622	0.500	0.308	0.145	0.082	0.028	0.022	0.018	0.014	0.013	0.012
	3	0.457	0.416	0.267	0.107	0.079	0.032	0.024	0.016	0.011	0.011	0.009
	4	0.595	0.458	0.309	0.161	0.099	0.076	0.067	0.045	0.037	0.022	0.019
	5	0.510	0.398	0.238	0.092	0.054	0.042	0.037	0.028	0.019	0.016	0.012

Qualitative Observations

- The skim milk has a bluish-tint due to Tyndall effect (Hewitt 2023)
- After 5 minutes, the solution became mostly transparent with a slight haziness as seen in Figure 4. Whilst somewhat clearer, it is still not as clear as distilled water.
- For 1% trypsin, there was more cloudiness observed in the cuvette after 5 minutes.
- 2%, 3%, 4%, and 5% did not display rippling cloudiness; all trials resembled very similarly
- Cloudy sediment at the bottom of every cuvette was observed.
- Absorbance fluctuated by 0.005AU sporadically.

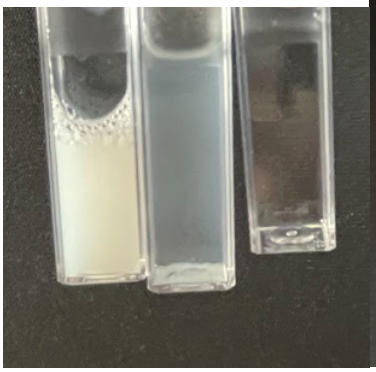

Figure 4: (Left to right) Milk solution, the solution after 5 minutes of 4% trypsin, distilled water

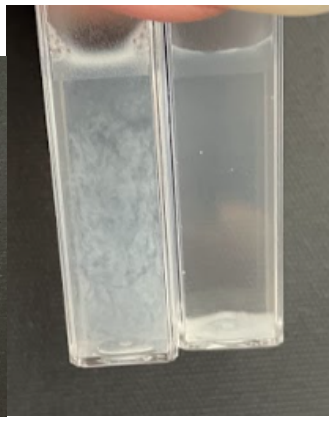

Figure 5: Milk solution with 1% trypsin (left), milk solution with 2% trypsin (right)

Processed Data:

Table 4: The Change in Absorbance of Milk, its Average, and the Rate of Hydrolysis and Its Average

Trypsin Conc. (±0.2%)	Trial	Change in Absorbance of Milk After 5 Minutes (±0.002 AU)	Average Change in Absorbance of Milk After 5 Minutes (±0.002 AU)	Rate of Hydrolysis (±0.0004 AU/min)	Average Rate of Hydrolysis (±0.0004 AU/min)
1	1	0.429	0.402	0.086	0.080
	2	0.453		0.091	
	3	0.409		0.082	
	4	0.355		0.071	
	5	0.365		0.073	
2	1	0.410	0.424	0.082	0.085
	2	0.440		0.088	
	3	0.486		0.097	
	4	0.344		0.069	
	5	0.442		0.088	
3	1	0.512	0.485	0.102	0.097
	2	0.465		0.093	
	3	0.537		0.107	
	4	0.435		0.087	
	5	0.475		0.095	
4	1	0.475	0.516	0.107	0.103
	2	0.536		0.094	
	3	0.517		0.103	
	4	0.526		0.105	
	5	0.533		0.107	
5	1	0.497	0.526	0.099	0.105
	2	0.610		0.122	
	3	0.448		0.090	
	4	0.576		0.115	
	5	0.498		0.100	

Sample Calculations:

All calculations were made on a CASIO FX-CG50 AU graphics calculator unless stated otherwise.

Calculating the rate of hydrolysis after 5 minutes:

$$\frac{|Change\ in\ absorbance\ after\ 5\ minutes|}{5} = Rate\ of\ Casein\ Hydrolysis$$

Therefore, the rate of hydrolysis for the first trial of 1% trypsin can be calculated, using data from Table 3:

$$\frac{|0.194-0.623|}{5} \approx 0.086\ AU/minute\ (\pm 0.0004\ \text{AU/minute})$$

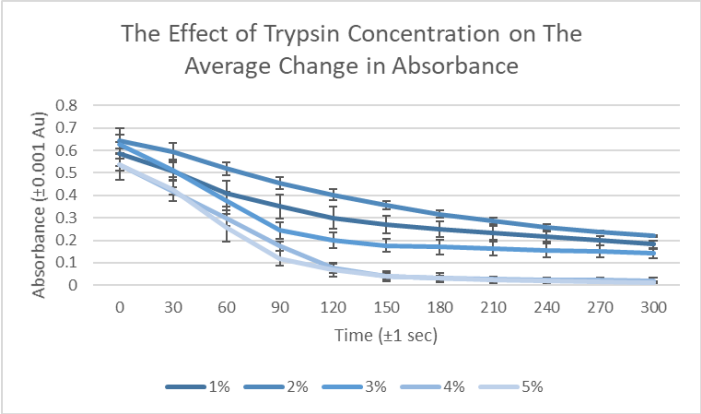

Figure 6: Graph depicting the effect of trypsin concentration on the average change in absorbance.
Error bars represent standard deviation, as calculated on Microsoft Excel 2016. Values are according to Table 4.

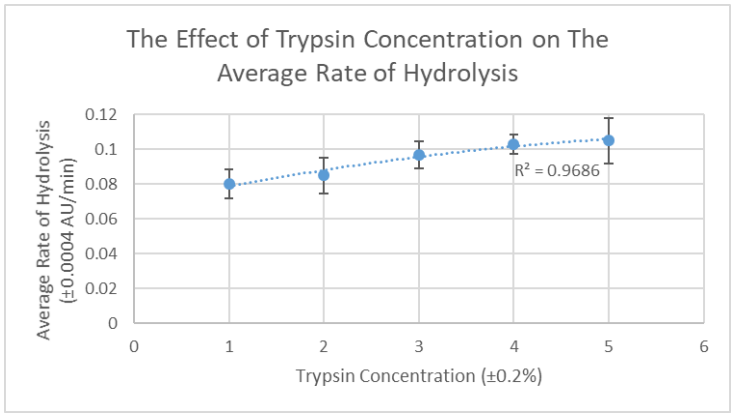

Figure 7: Graph depicting the effect of trypsin concentration on the average rate of hydrolysis.
Error bars represent standard deviation, as calculated on Microsoft Excel 2016. Polynomial trendline and R^2 value included. Values are according to Table 4.

Table 5: The percentage uncertainty of the smallest values of each trial (value at 300s), according to Table 3

Trypsin Conc. (±0.2%)	Percentage Uncertainty (%)					
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Average
1	0.515	0.508	0.613	0.602	0.526	0.430
2	0.457	0.461	0.444	0.467	0.455	0.457
3	0.627	0.862	0.794	0.694	0.602	0.716
4	6.667	4.545	2.500	9.091	5.882	5.737
5	9.091	8.333	11.111	5.263	8.333	8.426

As there are many data points within the five trials over five minutes, only the maximum percentage uncertainty is considered by selecting the smallest absorbance value of each trial. Due to the nature of this experiment, this is seen as the final absorbance value.

The percentage uncertainty is calculated using the following equation:

$$Percentage\ uncertainty\ = \frac{Absolute\ uncertainty\ of\ Colourimetry}{Recorded\ Experimental\ Data\ Value} \times 100$$

With the aforementioned equation, the maximum percentage uncertainty for Trial 1 with 1% trypsin can be calculated:

$$\frac{0.001}{0.194} \times 100 \approx 0.515\%$$

Table 6: The uncertainty propagation of each independent variable of the experiment and final absolute uncertainty

Trypsin Conentration (±0.2%)	Avg. %Uncertainty of Absorbance (±0.002 AU)	Avg. %Uncertainty of Trypsin Concentration (±0.2%)	%Uncertainty of Skim Milk Volume (±0.005ml)	%Uncertainty of Trypsin Volume (±0.005ml)	Total %Uncertainty	Absolute Uncertainty (±AU/min)
1	0.430	20.00	0.25	0.50	21.18	0.07
2	0.457	10.00	0.25	0.50	11.21	0.01
3	0.716	6.67	0.25	0.50	8.136	0.008
4	5.737	5.00	0.25	0.50	11.49	0.01
5	8.426	4.00	0.25	0.50	13.18	0.01

Similarly, the aforementioned equation is used to calculate the percentage uncertainty of each independent variable, adding them together to attain the total percentage uncertainty:

$$Total\ Percentage\ Uncertainty\ = \%Uncertainty_{Absorbance} + \%Uncertainty_{Tryps.Conc.} + \%Uncertainty_{Milk} + \%Uncertainty_{Trypsin}$$

The calculation for the total percentage uncertainty of 1% trypsin is as follows:

$$0.430 + 20 + 0.25 + 0.5 \approx \pm\ 21.18\%$$

Using the percentage uncertainty, the absolute uncertainty for the rate of hydrolysis can also be calculated:

$$Absolute\ Uncertainty\ = \frac{Total\ \%Uncertainty}{100} \times Rate\ of\ Hydrolysis$$

The calculation for the absolute uncertainty of the rate of hydrolysis from 1% trypsin is a follows:

$$\frac{21.18}{100} \times 0.08 \approx\ \pm\ 0.0169\ AU/min$$

Conclusion

It can be concluded from the results that the hypothesis is supported in that as the trypsin concentration increases, the rate of hydrolysis also increases (Robinson 2015; Liu 2017). The greatest trypsin concentration tested (5%) resulted in the greatest rate of hydrolysis at 0.105AU/min, as seen in Table 4 and Figure 7, and conversely, 1% trypsin resulted in the lowest rate of hydrolysis at 0.080AU/min. By increasing the concentration of trypsin by 4%, there is a 31.25% increase in the rate of hydrolysis. In Figure 6, the change in absorbance can be seen as a steeper decrease in absorbance as trypsin concentration increases; the plateau also occurs earlier and more prominently as trypsin increases.

Scientific Context

As colourimetry is based on Beer-Lambert's Law, it is assumed that there is a direct relationship between the absorption of light transmitted through a medium and its concentration (Arnold 2015). The hydrolysis of casein results in smaller and thus, more soluble, amino acids, which ultimately turns milk from a translucent white solution into a colourless liquid. By applying Beer-Lambert's Law, the change in the concentration of casein in milk can be quantified and used as an indicator of the hydrolysis of casein.

Increasing the concentration of trypsin leads to more trypsin molecules being present, which allows for more catalytic action. This is due to the increased number of substrate molecules that can bind to an enzyme. However, this is not an exactly linear relationship as the percentage increase of hydrolysis between 1% to 2% is greater than the percentage increase between 4% and 5%: (1.94%<6.25%). In Figure 5, 4% and 5% trypsin result in very similar plateaus, which is consequenced by all enzymes being fully saturated with casein molecules; enzymatic activity is limited by the number of enzyme-substrate binding sites (Cornell 2016). However, the rate of hydrolysis with 5% trypsin still exceeds 4% as the presence of more trypsin molecules increases the frequency of collisions.

Evaluation

Strengths

A significant strength of this experiment is its ease and simplicity, which would easily allow for the collection of more results to increase the precision of this experiment. Additionally, while animal-derived products are used, the method of colourimetry ensures that they are used extremely sparingly (2ml of milk per trial). This reduces both wastage and any environmental impact. The use of colourimetry also allows for both qualitative and quantitative results, providing a more comprehensive to this exploration.

Limitations

A significant limitation of this experiment is the cumulative deterioration which is also inherent in the use of enzymes as the various trials in this experiment were not completed simultaneously. Over time, trypsin is susceptible to self-catalysis due to its strong catalytic effect and therefore, some trials would have contained fewer or more active enzymes. Running multiple trials simultaneously will help to reduce the impact of cumulative deterioration in the future. Additionally, the experiment focuses on the hydrolysis of casein as indicated by the change in absorbance, however, other proteins and minerals are also present in milk. This may obstruct the colourimeter and lead to a final rate of reaction that plateaus due to residual components in milk, rather than due to enzymatic activity. For example, whey makes up around 20% of the protein found in milk with the majority being casein at 80% (Pikosky 2016). Whey is also hydrolysed although by measuring the change in absorbance, only casein is considered as it undergoes a colour change from white to colourless.

Additionally, as the aim of this experiment is to investigate enzymatic activity in relation to postoperative recovery and trauma, this experiment is limited due to its in vitro application. However, this approach may be used as an initial investigation to trypsin activity within laboratory conditions and as such, minimise both safety, ethical, and environmental risks.

Random Errors

The random errors that are present in this experiment are indicated by the error bars seen in Figures 6 and 7, and thus, represent the precision of the results.

As aforementioned, the natural degradation or 'death' of enzymes cannot be avoided and therefore, is likely to have influenced the rate of hydrolysis. For example, trial 4 of 2% trypsin may have possibly contained a higher concentration of inactive enzymes than other trials within the concentration, as indicated by the low change in absorption (see Table 4). This is especially true as it is the smallest change in absorbance in the entire data set, despite being the experimental result with 2% trypsin; theoretically, this trial should have yielded results greater than trials with 1% trypsin concentration. Due to the Collision Theory, this should not have been the case as 2% trypsin would have had double the number of trypsin molecules than 1% (Jung et al. 2014).

- Improvement: immobilised trypsin may be used to prolong the stability and longevity of the enzyme and to reduce the amount of enzyme inactivity.

There was a lack of temperature control as consequenced by the colourimeter; the device cannot be submerged in a water bath or placed on a heating plate (see Table 2). As a result, fluctuations in temperature may have affected enzymatic activity as the temperature can accelerate or denature enzymes due to low or a surplus kinetic energy, respectively (Daniel et al. 2007). However, as all materials were contained in an air-conditioned room, these fluctuations would have been consistent across all materials and apparatus.

- Improvement: a greater number of trials may be performed simultaneously in an air-conditioned room to optimise temperature similarity across all trials. By collecting data simultaneously, the same temperature will be present across all trails, ensuring that it is a controlled experiment. Ensure that there is minimal traffic in the area to prevent temperature fluctuation.

Systematic Errors

As milk is not a source of 100% casein, there are other minerals and potential impurities that obscure its absorbance. While there is an inverse correlation between trypsin concentration and the final absorbance at 300s, this is evidenced by Table 4 whereby the absorbance never reaches 0AU although plateaus, indicating a limit to enzyme-substrate binding. Figures 3 and 4 also show the residual cloudiness, especially in 1% trypsin, that remains. While translucence increases, the impurities continue to obstruct the path length of light through the solution. This ultimately affects the recorded data as absorbance is correlated with concentration, according to Beer's Law, which states that the light absorption that passes through a medium is directly proportional to the concentration of the medium. While the true

concentration of casein may be very low in every data point, it will always be higher, to some extent, as impurities continue to interrupt the device's light.

- Improvement: while milk is a readily available and fairly inexpensive source of casein, for a more accurate recording of casein hydrolysis, a solution of pure casein isolate should be used. This removes any impurities that may obstruct the true absorption value.

Another systematic error that was present was the calibration of the colourimeter, as the device had a hole in the bottom, which could have allowed some extraneous light to enter. This was made apparent by the fluctuating absorbance that was observed during the data collection. While the device was calibrated prior to use, this fluctuation still occurred which indicates the presence of some inaccuracy within the results.

- Improvement: as the Vernier Colourimeter may allow some external light to enter the device through the base hole, a colourimeter without such a cavity should be used instead. This will ensure accurate calibration and thus, more reliable results.

General Improvements

As random errors are present in this experiment, more trials would allow for a more precise result. This will ensure that the effect of anomalies consequenced by random errors will be minimised and therefore, the final data will be deemed more reliable. A strength of this experiment is its simplicity and fairly quick method of data collection which allows for efficient trial repetition. Additionally, by having more trials within a short time frame the effect of protein degradation will also be minimised. Furthermore, the use of porcine trypsin and cow's milk poses the ethical concern of using animal-derived products within this practice. Recombinant enzymes and non-animal substrates may be considered in the future to reduce the ethical implications of this experiment. Using a method of data collection that allows the use of temperature control (ie. water bath, heating pad) will also help to control an extraneous factor that would influence enzymatic activity.

Extension

This experiment's simple methodology can be adapted and applied to a variety of enzymatic investigations and the effect of other factors such as temperature, substrate concentration, pH, and inhibitors. As organic catalysts with specialised roles, all enzymes occupy different optimum conditions which ultimately affect their functions and processes. For example, the effect of amylase concentration can be investigated in conjunction with starch as its respective substrate; the hydrolytic process of starch by amylase is akin to the hydrolysis of casein by trypsin. As the initial aim of this experiment was to investigate the processes of enzymes and biochemical reactions, investigating other enzymatic factors will allow for a more comprehensive understanding and application for industrial use.

Bibliography

Arnold, B. (2015) *The Working Principle of Colorimeters*, *AZO*. Available at: https://www.azosensors.com/article.aspx?ArticleID=324#:~:text=The%20colorimeter%20is%20based%20on,proportional%20to%20the%20medium%20concentration (Accessed: 30 May 2023).

Cell Dissociation Protocol using Trypsin (2023) *Merck*. Available at: https://www.sigmaaldrich.com/AU/en/technical-documents/protocol/cell-culture-and-cell-culture-analysis/mammalian-cell-culture/cell-dissociation-with-trypsin (Accessed: 30 May 2023).

Colorimeter – Definition and Uses of Colorimeter (2023) *TOPPR*. Available at: https://www.toppr.com/guides/chemistry/solutions/colorimeter-definition-and-uses-of-colorimeter/ (Accessed: 25 June 2023).

Cornell, B. (2016) *Enzyme Catalysis*, *Bioninja*. Available at: https://www.google.com/url?q=https://ib.bioninja.com.au/standard-level/topic-2-molecular-biology/25-enzymes/enzyme-catalysis.html&sa=D&source=docs&ust=1685442800383231&usg=AOvVaw1LUyTf1uFEV6GE4v5Rv8vP (Accessed: 30 May 2023).

Daniel, R. *et al.* (2007) *The effect of temperature on enzyme activity: new insights and their implications*, *SpringerLink*. Available at: https://link.springer.com/article/10.1007/s00792-007-0089-7 (Accessed: 30 May 2023).

Davis, J. (2021) *What are Zwitterions?*, *AZO*. Available at: https://www.azolifesciences.com/article/What-are-Zwitterions.aspx#:~:text=Every%20zwitterion%20has%20an%20isoelectric,is%20considered%20to%20be%20basic (Accessed: 30 May 2023).

Helmenstine, A. (2019) *Why Milk Is White*, *ThoughtCo.* Available at: https://www.thoughtco.com/why-milk-is-white-606172#:~:text=Fat%2Dfree%20or%20skim%20milk,more%20blue%20light%20than%20red (Accessed: 30 May 2023).

Haas, K. (2020) *8.1.1: Colors of Coordination Compounds (Electronic Absorption Spectra)*, *LibreTexts*. Available at: https://chem.libretexts.org/Courses/Saint_Marys_College_Notre_Dame_IN/CHEM_342%3A_Bio-inorganic_Chemistry/Readings/Week_8%3A_LFT_continued...Electronic_Properties/8.1%3A_Absorption_Spectra_and_Magnetic_Properties/8.1.1%3A_Colors_of_Coordination_Compounds_%28Electronic_Absorption_Spectra%29 (Accessed: 28 June 2023).

Hewitt, S. (2023) *Why is milk white?*, *Fonterra*. Available at: https://www.fonterra.com/nz/en/our-stories/articles/why-is-milk-white.html#:~:text=Not%20to%20worry%2C%20not%20all,%2C%20such%20as%20beta%2Dcarotene (Accessed: 30 May 2023).

Johnson, K. and Marshall, A. (2002) *A functional comparison of ovine and porcine trypsins*. Available at: https://pubmed.ncbi.nlm.nih.gov/11959024/ (Accessed: 25 June 2023).

Jung, K.-H. *et al.* (2014) *Effects of Concentration and Reaction Time of Trypsin, Pepsin, and Chymotrypsin on the Hydrolysis Efficiency of Porcine Placenta*, *National Library of Medicine*. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4597853/ (Accessed: 30 May 2023).

Liu, S. (2017) *Enzyme Concentration*, *ScienceDirect*. Available at: https://www.sciencedirect.com/topics/engineering/enzyme-concentration (Accessed: 30 May 2023).

Margraf, A. *et al.* (2020) *Systemic Inflammatory Response Syndrome After Surgery: Mechanisms and Protection*, *National Library of Medicine*. Available at: https://pubmed.ncbi.nlm.nih.gov/33186158/#:~:text=Surgical%20procedures%20thus%20represent%20a,tissue%20and%20multisystem%20organ%20destruction (Accessed: 30 May 2023).

Robinson, P.K. (2015) *Enzymes: principles and biotechnological applications*, *National Library of Medicine*. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4692135/#:~:text=The%20relationship%20between%20enzyme%20concentration,will%20proceed%20twice%20as%20fast (Accessed: 30 May 2023).

Scripps, A. (2023) *StableCellTM Trypsin Solutions*, *Merck*. Available at: https://www.sigmaaldrich.com/AU/en/technical-documents/protocol/cell-culture-and-cell-culture-analysis/mammalian-cell-culture/stablecell-trypsin-solutions (Accessed: 30 May 2023).

Sethi, S. (2018) *Trypsin Function*, *Healthline*. Edited by S. Frothingham. Available at: https://www.healthline.com/health/why-are-enzymes-important#takeaway (Accessed: 30 May 2023).