sserc PROTOCOL

INVESTIGATING TRYPSIN ACTIVITY IN MILK

Background

Trypsin is secreted by the pancreas as trypsinogen, and activated in the small intestine. It hydrolyses proteins to smaller, soluble peptides and amino acids. Casein, a protein found in milk, is broken down by trypsin to form small peptides. This can be monitored experimentally in the classroom - as trypsin activity proceeds, a milk suspension becomes clearer.

In this protocol, it is likely porcine pancreas trypsin will be used (e.g. Philip Harris supplies this). This gives learners an opportunity to compare the primary structures of pig (accession number: P00761) and human (accession number: P07477) trypsin. A primary sequence alignment between these two proteins reveals 79% sequence identity (Figure 1).

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CLUSTAL O(1.2.4) multiple sequence alignment

sp|P07477|TRY1_HUMAN      MNPLLILTFVAAALAAPFDDDDKIVGGYNCEENSVPYQVSLNSGYHFCGGSLINEQWVVS  60
sp|P00761|TRYP_PIG        ---------------FPTDDDDKIVGGYTCAANSIPYQVSLNSGSHFCGGSLINSQWVVS  45
                                        * **********.*  **:********* *********.*****

sp|P07477|TRY1_HUMAN      AGHCYKSRIQVRLGEHNIEVLEGNEQFINAAKIIRHPQYDRKTLNNDIMLIKLSSRAVIN  120
sp|P00761|TRYP_PIG        AAHCYKSRIQVRLGEHNIDVLEGNEQFINAAKIITHPNFNGNTLDNDIMLIKLSSPATLN  105
                          *.****************:*************** **::: :**:********** *.:*

sp|P07477|TRY1_HUMAN      ARVSTISLPTAPPATGTKCLISGWGNTASSGADYPDELQCLDAPVLSQAKCEASYPGKIT  180
sp|P00761|TRYP_PIG        SRVATVSLPRSCAAAGTECLISGWGNTKSSGSSYPSLLQCLKAPVLSDSSCKSSYPGQIT  165
                          :**:*:*** :  *:**:********* ***:.**. ****.*****::.*::****:**

sp|P07477|TRY1_HUMAN      SNMFCVGFLEGGKDSCQGDSGGPVVCNGQLQGVVSWGDGCAQKNKPGVYTKVYNYVKWIK  240
sp|P00761|TRYP_PIG        GNMICVGFLEGGKDSCQGDSGGPVVCNGQLQGIVSWGYGCAQKNKPGVYTKVCNYVNWIQ  225
                          .**:****************************:**** ************** ***:**:

sp|P07477|TRY1_HUMAN      NTIAANS       247
sp|P00761|TRYP_PIG        QTIAAN-       231
                          :*****
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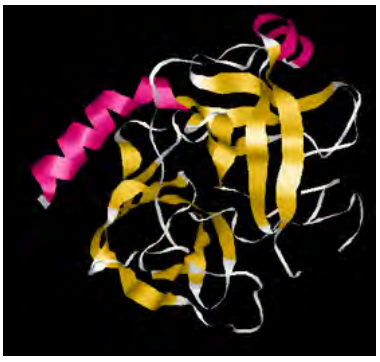
A BLAST alignment between human and pig trypsin shows a high level of homology / conservation providing justification for using pig trypsin to make inferences about the human enzyme. Uniprot was used to find these sequences.



Human trypsin (protein data bank: 1TRN) can be viewed using RasMol to reveal its secondary and tertiary structure (as shown opposite). Depending on the curriculum level, this can provide opportunities to explore structural details of a specific protein and provides significant depth within a Project Introduction at Advanced Higher Biology level.

Trypsin activity provides a specific insight into pancreatic health because it is only released from here. In addition to its digestive function, trypsin has also been shown ot have a role in regulating blood pressure, inflammatory processes and in the pathogeneis of neurodegenerative diseases. Understanding the function of trypsin, a relatively under-researched enzyme, is important because of the growing prevalance of pancreatic disease worldwide (Vertiprakhov, 2022).

References

Vertiprakhov, V.G. and Ovchinnikova, N. V. (2022), *The activity of trypsin in the pancreatic juice and blood of poultry increases simultaneously in the postprandial period*, Frontiers in Physiology, 13:874664. Available here: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9641294/. Accessed 01/11/2023.

AIM

Aim: To investigate the effect of temperature on trypsin activity.

This protocol can be adapted to investigate:

- effect of pH on trypsin activity
- effect of substrate concentration
- effect of enzyme concentration

Two alternative methodologies will be presented for this protocol. The first protocol might be more appropriate from National 5 Biology, while the second might suit a Higher/Higher Human Biology class.

PROTOCOL 1: HOW LONG DOES IT TAKE FOR THE X TO DISAPPEAR?

Materials: The volumes stated in the table below are exact volumes per group. Provide a little extra for each group to allow for pipetting errors. This allows for triplicate measurements across 5 temperatures, with 1 control per temperature.

200 cm^3 3% milk suspension (powdered)	6 test tubes
50 cm^3 pH 7.0 buffer	Stopwatch
30 cm^3 1% trypsin solution	Marker pen
Thermostatically-controlled waterbath	3x syringes / pipettes

1. Start with the lowest temperature. Collect 2 test tubes and label with "C" (control) or "T" (test). Use a marker pen to draw a X about half-way up each test tube (*image 1*).

Image 1: Draw an X on each test tube.

2. Pre-incubate the enzyme, buffer and milk suspension for about 5 minutes (*image 2*).

3. Using separate syringes / pipettes, set up the test tubes as shown in I*mage 3*. Add the enzyme last and then start the stopwatch.

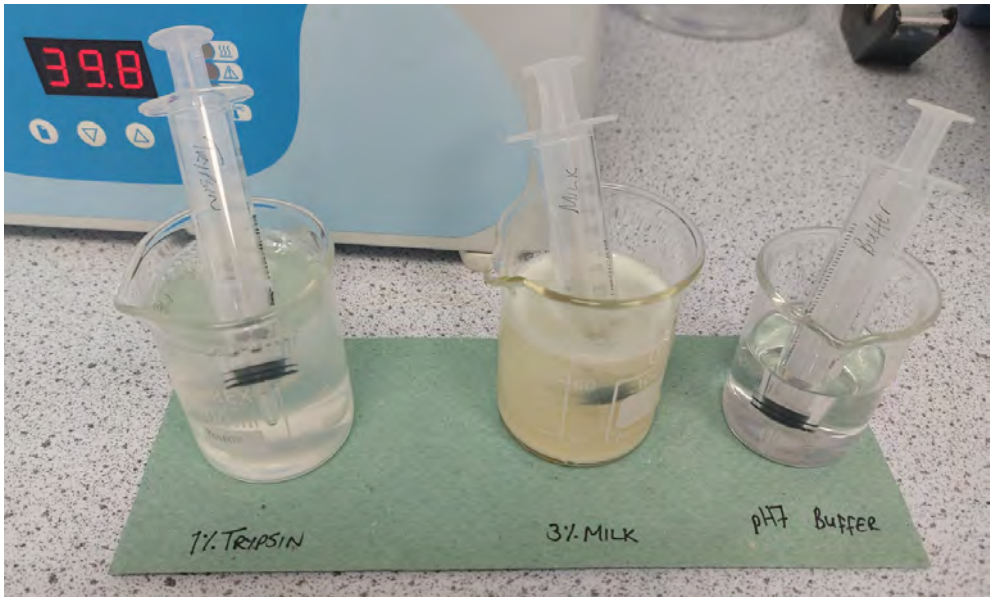


Image 2: Reagents equilibrating to room temperature for the lowest temperature point.

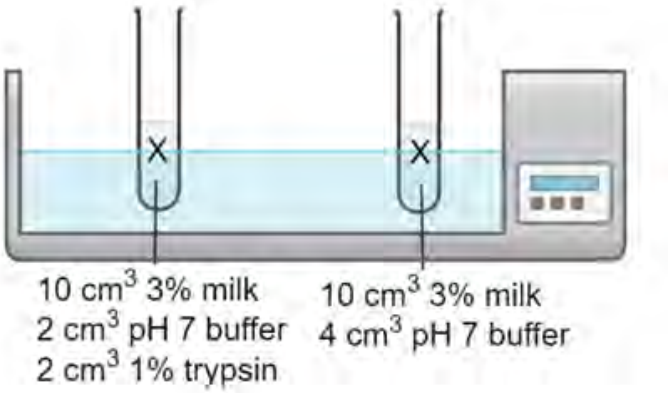


Image 3: Volumes of each reagent required in the "Test" and "Control" test tubes.

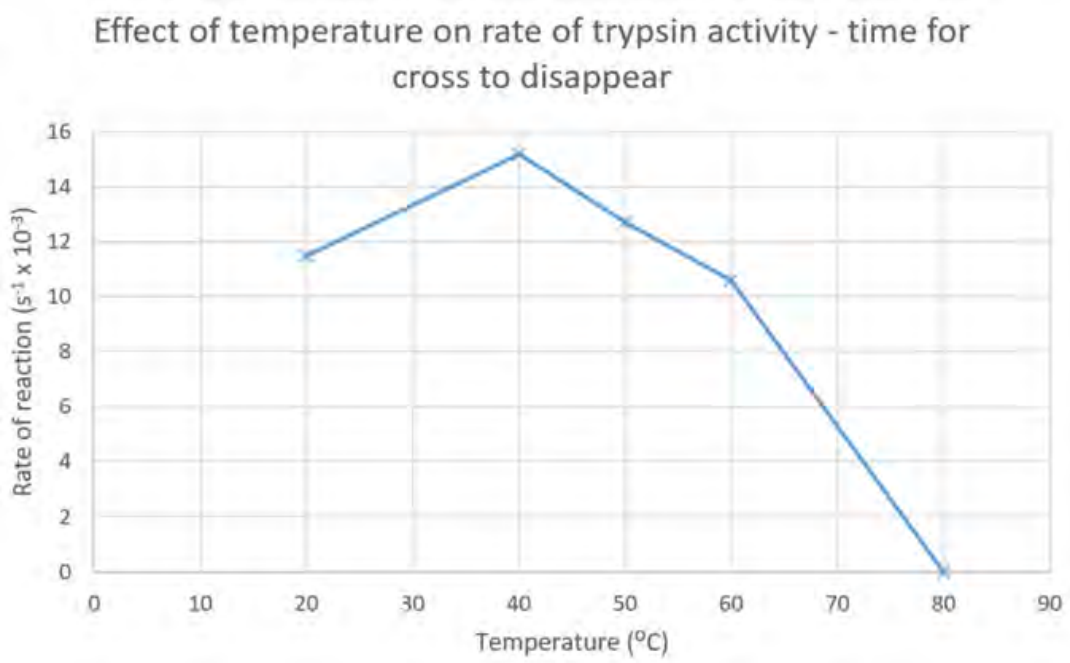
4 After trypsin has been added to the "Test" test tube, record the time it takes for the X to become visible from the opposite side of the test tube (*image 4*).

Image 4: The "Test" reaction is on the left, containing trypsin. After a period of time, the X becomes visible through the glass. This does not happen for the "Control" reaction lacking enzyme.

SAMPLE RESULTS

The table below show mean results from triplicate experiments at five different temperatures. The rate of reactio (1/time taken for X to disappear (s) was noted. At 80 °C, no clearing took place after five minutes, with no obser difference between the Test and Control reactions.

Temperature (°C)	Time taken for X to disappear (s)				Rate of reaction (1/s)	Rate of reaction (s^{-1} x 10^{-3})
	1	2	3	Mean		
20	85	90	86	87	0.011	11.5
40	60	75	62	66	0.015	15.2
50	75	80	82	79	0.013	12.7
60	90	95	98	94	0.011	10.6
80	No clearing	No clearing	No clearing	-	0	0



PROTOCOL 2: TRANSMISSION OF LIGHT THROUGH THE SAMPLE

Materials: The volumes stated in the table below are exact volumes per group. Provide a little extra for each group to allow for pipetting errors. This allows for triplicate measurements across 5 temperatures, with 1 control per temperature.

40 cm^3 3% milk suspension (powdered)	3 cuvettes
12.5 cm^3 pH 7.0 buffer	Stopwatch
7.5 cm^3 1% trypsin solution	Colorimeter
Thermostatically-controlled waterbath	3x syringes / pipettes
water	

1. Pre-incubate the enzyme, buffer and milk suspension at the lowest temperature for about 5 minutes.

2. Start with the "Control" reaction. Blank the colorimeter (red diode) on transmission mode, using a cuvette of water. Then set up the "Control" cuvette as shown in Image 5 below. Place the cuvette into the colorimeter, record the transmission reading, and start the stopwatch. Leave the reaction for 2 minutes and then record the transmission value. Calculate how much the transmission changed over 2 minutes. This will increase as the milk powder will start to settle - this value must be subtracted from the "Test" reaction.

3. With a clean cuvette, set up the "Test" reaction as shown in Image 5, leaving out the enzyme/trypsin. Place the cuvette in the colorimeter, add the enzyme volume, start the stopwatch and record the initial transmission value. After 2 minutes, record the transmission value again. Calculate the change in transmission and then subtract the change in transmission that took place in the "Control" tube over the same time.

4. Carry out the experiment in triplicate at each temperature, and then across all five suggested temperatures.

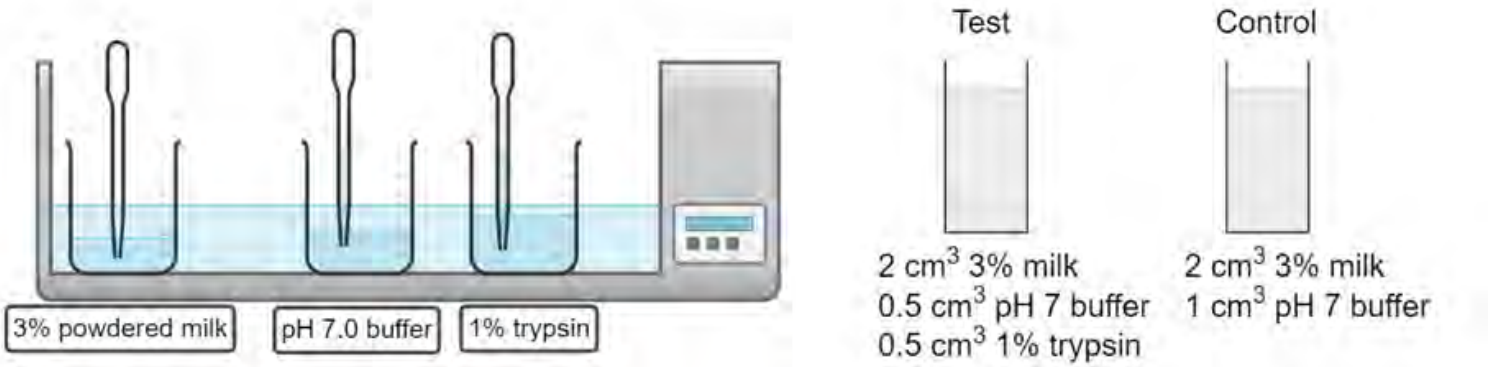


Image 5: Set up the Test and Control reactions as shown below, ensuring all reagents have equilibrated to the appropriate temperature for 5 minutes before use.

SAMPLE RESULTS

Control Reaction - *How did the transmission of light through the sample change over 120 s in the absence of trypsin at each temperature*

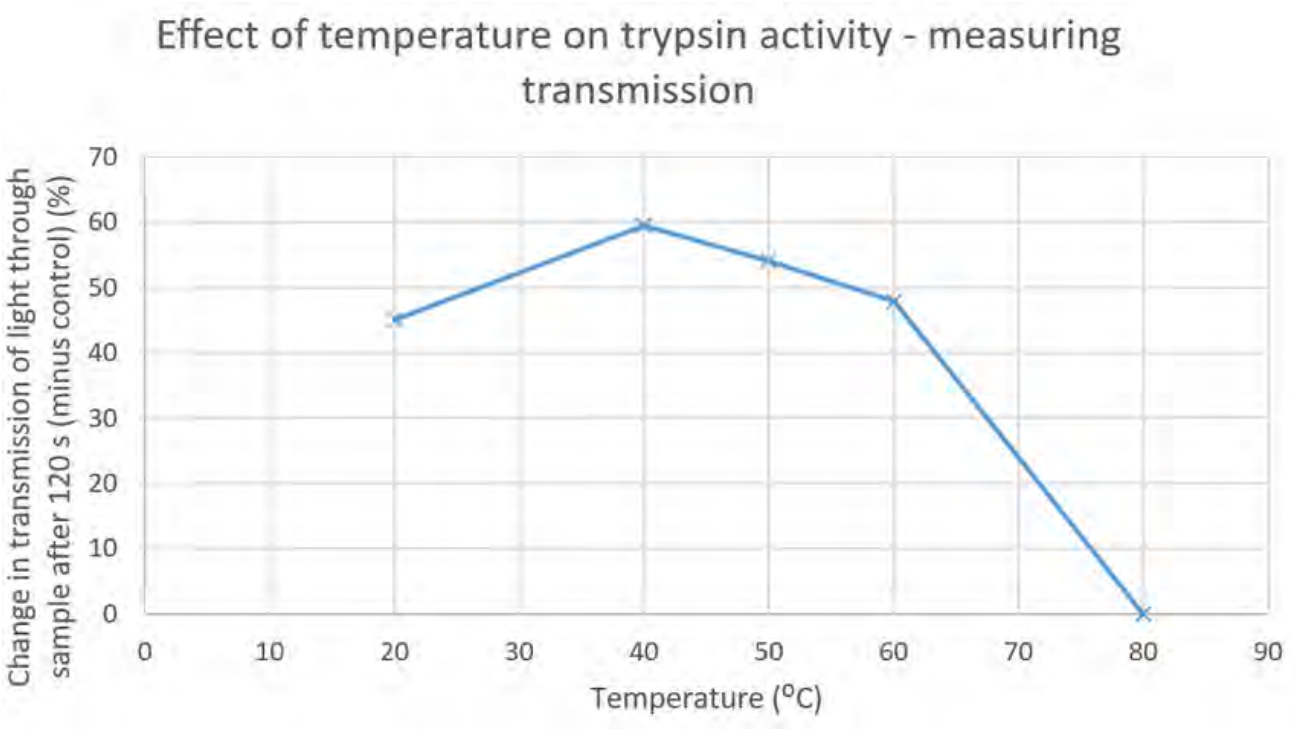
Temperature (C)	Transmission at 0 s	Transmission at 120 s	Change in transmission after 120 s
20	27	31.8	4.8
40	18.6	22.9	4.3
50	26.8	33.6	6.8
60	27	39.8	12.8
80	27	38	11

Test Reaction - *How did the transmission of light through the sample change over 120 s in the presence of trypsin at each temperature*

Temperature (C)	Transmission at 0 s	Transmission at 120 s	Change in transmission after 120 s
20	27.2	77	49.8
40	16.2	80.0	63.8
50	26.1	87.0	60.9
60	28.4	89.1	60.7
80	21.2	31.9	10.7

Corrected Results - The change in transmission after 120 s in the ***Control*** reaction were subtracted from the ***Test*** results to produce this final results table.

Temperature (C)	Transmission at 0 s	Transmission at 120 s	Change in transmission after 120 s	Corrected change in transmission after 120 s
20	27.2	77	49.8	45
40	16.2	80.0	63.8	59.5
50	26.1	87.0	60.9	54.1
60	28.4	89.1	60.7	47.9
80	21.2	31.9	10.7	0



CONCLUSION

Using either methodology, the results show that optimum trypsin activity can be observed at 40 °C. Evidence of denaturation was observed at 80 °C.

REAGENTS & MATERIALS

Trypsin: Can be purchased from Philip Harris (25 g) - £31.44 (inc. VAT). Product code: B8A01969 (Oct 2023). It can also be bought from Timstar (25 g) for £35.64 (inc. VAT). Code: EZ81581.

Skimmed powdered milk is required for this experiment. SSERC purchased this from Asda.

For Protocol 2, a **Mystrica colorimeter** was used. This can be purchased from Timstar, £151.20 (inc.VAT), product code: CO100720. SSERC gave 9 to each local authority between 2015 and 2016.

SSERC used a ready-made pH 7.0 buffer. Timstar supplies these (product code PH120125, £19.38 inc. VAT for 10 capsules, each to make up 100 ml buffer). Philip Harris supply 50 ml pH 7 buffer solutions for £5.88 (inc. VAT).