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Measuring the action of washing product proteases

An easy way to monitor protease activity

Proteases in 'biological' washing powders

At the start of the 20th century, German chemist Otto Röhm was trying to solve an unpleasant problem. He was trying to develop an improved method of 'bating', the process by which animal hides are softened before tanning to make leather. The motivation behind this work was that the substance then used for bating was highly offensive — dog faeces.

Dr. Rohm found that the active components in dog faeces were proteases — enzymes that degrade proteins. He reasoned that extracts from animal organs that produce similar digestive enzymes might be used instead. Fortunately for the tannery workers Rohm's investigations proved successful and by 1905 pig and cow pancreases were providing a more pleasant and reliable source of enzymes. To produce and sell the enzyme preparation Röhm teamed up with businessman Otto Haas to form the company *Röhm & Haas* in 1907. The pancreatic enzyme extract, which the firm called *Oropon*®, quickly became popular in the leather industry and *Röhm & Hass* followed this success with a range of other enzyme products.

World's first

Röhm continued his experiments with animal enzymes and in 1913 he was granted a German patent for the use of trypsin, extracted from animal pancreases, to clean soiled clothes. This is how Röhm explained his idea:

> *"As is known, tryptic enzymes have the ability to break down fat and protein. On the assumption that the main part of dirt in fabrics used by humans is composed of fat and protein residues, tryptic enzymes were added to the wash. It appeared that when this was done, the fabric could be cleaned in a shorter time, with less exertion of strength* and at a temperature far below the boiling point of water. Further, the fabric had a better appearance and much less soap was necessary. The advantage of using enzymes instead of other ingredients, especially alkaline chemicals, is that they do not attack the textile fibres..."*

In the same year that the patent was issued, *Röhm & Haas* started to market the world's first enzyme detergent, which they called *Burnus*®. Unfortunately the product was not a great success, and it is doubtful whether it worked very well. Its main components were washing soda

* *Röhm was writing at a time when most washing was done by hand and required physical strength.*

(sodium carbonate) plus a small amount of pancreatic extract. The washing soda made the solution very alkaline, and this would have reduced both the activity and stability of the enzymes. Röhm died in 1939, so it was left to others to develop the use of enzymes in washing products further.

Soap shortage

Major developments were next initiated in Switzerland by Dr. E. Jaag. Jaag had been inspired by Röhm's ideas when he started work in the 1930s and later by a wartime article proposing that enzymes could be used for cleaning clothes instead of soap. [During World War II, there was a severe shortage of fats (and consequently soap) in Europe, so enzyme-containing washing products could have assumed greater importance.]

However, Jaag had to wait until the war was over before he could make further progress, since all animal pancreases at the time were reserved for the production of insulin for treating diabetes.

Jaag's first product, *Bio38*, contained pancreatic trypsin with added bile salts to emulsify any fats. In 1947, he pointed out the advantages of using an enzyme product, but was also careful to indicate the potential disadvantages of using it to wash proteinaceous textiles *e.g.*, wool and silk. Jaag also noted that if such products were to have a future, new enzymes would have to be found that were tolerant of the alkaline conditions found in washing solutions.

Bacterial proteases

During World War II, much effort had been directed towards growing microorganisms in bulk. The fungus that makes penicillin requires oxygen for its growth, and initial attempts to produce penicillin for medical use had been based on surface culture in thousands of small containers including, at one time, bed-pans and milk bottles. A major advance was heralded by the development of fermenters in which the microbes were submerged in liquid medium and aerated with bubbles. These fermenters (sometimes referred to as Continuous Stirred Tank Reactors) allowed the industrial-scale production of microorganisms, for antibiotic production and subsequently the cultivation of microbes for enzyme production.

In 1959, Jaag, in collaboration with the *Swiss Ferment Company*, launched an improved product, *Bio40*. This contained a bacterial protease which worked best under neutral conditions. Although this was better than pancreatic enzymes, it was still not an ideal, since its stability in the alkaline conditions of the typical wash (pH 9–10) was too low.

While this was taking place, independent research in Copenhagen by *Novo Industri* (now *Novozymes*) was begun. The Danes' aim was to develop an enzymatic treatment for heavily-soiled overalls from the fish processing industry. Not only were the stains difficult to remove, but fishy odours emanated from the freshly-cleaned clothes as they were being ironed. By 1961 *Novo* had succeeded in producing a bacterial protease from *Bacillus licheniformis* that remained active and stable in alkaline washing conditions. Consequently the enzyme was given the trade name *Alcalase®*.

The associated washing treatment, a six-step procedure, required that the protein stains on the clothes were denatured in very hot (85 °C) detergent solution before the enzyme was added. Although the method gave excellent results compared with those normally used in industrial laundries, it was too complex to be widely adopted.

Confident in the knowledge that they had a better product than the *Swiss Ferment Company's* neutral protease, *Novo* approached Jaag's company, *Gebrüder Schnyder*, and signed an agreement to incorporate *Alcalase®* into *Bio40*. However, local competition from soap manufacturers prevented *Bio40* from becoming a success, so *Novo* and *Schnyder* entered into collaboration with a Dutch and a Danish company to produce and market an enzyme powder in Holland. In 1963 *Bio-tex* was launched, and within a short time it had captured 20% of the Dutch washing powder market.

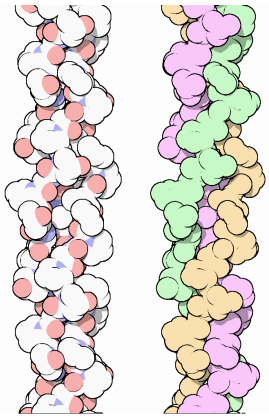
Since the introduction of optical brighteners into washing powders in the 1950s, the detergent business had remained more or less static. The sudden loss of a substantial proportion of the market to a newcomer aroused the attention of the major detergent manufacturers, who began to look at enzymes with renewed interest. Fifty years after Röhm's original patent, enzyme washing powders began to take off.

Today enzymatic detergents occupy 85% of the washing powder market in Western Europe and 50% of the market worldwide. Biological washing powders account for almost a quarter of the enzymes sold throughout the world. Most European consumers regard enzymes as beneficial and 'green', largely because of their effectiveness at low temperatures.

About gelatine

Gelatine (also spelt 'gelatin') is obtained from animals: usually pigs, cows or fish. Free gelatine molecules don't exist in meat or fish however. Gelatine is a breakdown product of a fibrous protein called collagen. Collagen gives mechanical strength to muscles, tendons and skin. When combined with minerals, it forms bones and teeth. Between 25% and 35% of the protein in a mammal's body is collagen.

Each collagen molecule is formed from three polypeptide strands bound together in a triple helix. Gelatine is made by partially breaking down collagen. When collagen is heated to approximately 60 °C, the strands of the triple helix start to separate. Further heating may cause the strands to break up into shorter polypeptides. When the solution is cooled down, the strands do not reform into collagen, but form a mass of tangled polypeptide chains, trapping water inside. Gelatine gel, with added sugar and flavouring, is familiar as a dessert and in confectionary.



A small portion of collagen, coloured by atom (left) and coloured to highlight the three chains (right). From: David Goodsell. April 2000. Molecule of the month: Collagen. doi: 10.2210/rcsb_pdb/mom_2000_4.

Aim

This simple technique allows protease activity to be monitored by measuring the diameter of the wells produced in a Petri dish of gelatine. The basic procedure can be adapted for use with proteases from a wide variety of sources including plant and microbial enzymes. It is however, unsuitable for investigating the effect of temperature above about 30 °C, as gelatine will start to melt at this temperature.

Equipment and materials

Needed by each person or working group

Equipment

- wide-bored plastic drinking straw for cutting wells in the gelatine
- mounted dissecting needle or a cocktail stick for removing the gelatine plugs if necessary
- permanent marker pen, fine-tipped, for labelling the Petri dish
- ruler for measuring the diameter of the wells after incubation

Materials

- suitable proteases *e.g.*, *Neutrase®*, *Alcalase®*, *Savinase®* (approximately 1 drop per test)
- Petri dish containing set gelatine

Procedure

Decide how many enzymes you are going to test and what 'controls' will be necessary. You will need to cut a well in the gelatine for each treatment. Remember to space the wells widely apart because the enzymes, if they break down the gelatine, will cause the wells to enlarge.

1 Use the drinking straw to cut wells in the gelatine in the Petri dish. If you rotate the straw carefully as you make each well, it will usually cut a neat hole, and the plug of gelatine will stick inside the straw as you lift it. If the gelatine plug doesn't lift away with the straw, very carefully use a mounted needle of cocktail stick to lift out the plug.

2 Turn the Petri dish over and write on the base so that each well is labelled and you will know what has been added to it. Also write your initials and the date so that you can identify your plate.

3 Add approximately one drop of enzyme or other test substance to each well.

4 Put the lid on the Petri dish and leave it undisturbed for up to 24 hours.

5 After 24 hours, measure the diameter of the wells in the gelatine
*Note: you may need to wash the gelatine **very gently** with **cold** water to obtain a better view of the wells.*

Teacher's and technician's notes

Preparation and timing

You will need to prepare the gelatine plates several hours in advance. Although it takes only minutes to pour each plate, the gelatine will take several hours to set, so it is safer to prepare the plates at least the day before the practical activity.

The practical activity itself takes about 30 minutes, followed by an incubation period of 1–24 hours.

Preparing the gelatine plates

It is best to use bovine (cow) gelatine rather than porcine (pig) gelatine, because the latter is cheaper, easier to prepare and generally sets to a firmer gel. The most widely available brand of bovine gelatine available in the UK is *Dr Oetker* (usually found in the cake-making section of a supermarket). This costs £1 for 3 × 12 g sachets (November 2016 price). One sachet, prepared with approximately *a quarter* the volume of water suggested on the packet, will be sufficient for 5 Petri dishes.

To prepare the gelatine, boil some tap water and add 150 cm^3 to a jug. Allow the liquid to cool slightly if it is still very hot. Sprinkle the contents of one 12 g sachet of gelatine evenly over the surface of the water, stirring the liquid as you do so. Keep stirring until no lumps remain. Do not boil the solution, as this will degrade the gelatine and prevent it from setting firmly.

Add approximately 1 cm^3 of food colouring to the solution and mix well: dark colours are best, but don't add too much and avoid black colouring as this will make it impossible to see any labels written on the Petri dish. The colour will allow students to see the wells in the gelatine more easily, but if you add too much colouring, as the well enlarges, colour will seep into the well and make the edges of it difficult to see.

Dispense the gelatine into Petri dishes and leave them covered in a cool place or ideally, a fridge to set. Note that you need approximately 30 cm^3 of gel in each Petri dish to obtain deep enough wells — more than you would typically use for microbiology media. It will take at least two hours for the gelatine to set.

Safety

Enzymes

Please refer to the Association for Science Education publication *Topics in Safety* (2016). Chapter 20, 'Working with enzymes' is available from both the NCBE and the ASE web sites.

Equipment and materials

Needed by each person or working group

Equipment

- wide-bored plastic drinking straw (approximately 4 mm diameter) for cutting wells in the gelatine
- mounted dissecting needle or a cocktail stick for removing the gelatin plugs if necessary
- permanent marker pen, fine-tipped, for labelling the Petri dish
- ruler for measuring the diameter of the wells after incubation

Materials

- suitable proteases *e.g.*, *Neutrase®*, *Alcalase®*, *Savinase®* (approximately 1 drop (~50 µL) per test)
- Petri dish containing approximately 30 cm^3 of set gelatin (see recipe above)

Troubleshooting

The wells enlarge too much
If you intend leaving the plates for 24 hours or more, you will probably find that the enzyme needs diluting considerably (say, 10-fold) so that it will give reasonable results over that extended incubation period (you could also try keeping the plates in a fridge to slow down the enzyme action). Conversely, if you want the investigation to take only 60 minutes or so, use the enzymes neat from the bottle. It is difficult to give precise guidance here, as much will depend upon the gelatine concentration and the room temperature.

The gelatine liquifies
Gelatine gels will melt at ~30 °C, and even at 20 °C they can go soft. If the weather is warm, put the plates in a fridge after the enzymes, *etc.* have been dropped into the wells. The enzyme activity will be slower, but the risk of the gel melting will be removed.

Additional investigations

There is limited scope for additional investigations here as altering the pH and/or temperature may cause the gelatine to liquefy. Students can, however, compare the action of different proteases, and the effect of enzyme concentration (a possible opportunity for serial dilution).

Enzyme specificity can also be demonstrated by adding enzymes other than proteases in the wells. The importance of 'controls' can also be emphasised.

Reference

David S. Goodsell. April 2000. Molecule of the month: Collagen
https://pdb101.rcsb.org/motm/4

Suppliers

Proteases

Suitable proteases are available from the NCBE: these include *Savinase®* and *Alcalase®*. Extracts from plant materials may also be tested for protease activity: kiwi fruit and pineapple are good sources.

Gelatine

Suitable gelatine is widely available from supermarkets and online. In the UK, powdered gelatine is generally derived from cows, whereas the more costly leaf gelatine is from pigs. Powdered bovine gelatine is easier to prepare and forms a firmer gel. We have achieved successful results with the *Dr Oetker* brand of powdered gelatine, which is widely available from UK supermarkets.

Most fruit jellies sold in the UK are also made with porcine gelatine or with a vegatarian substitutes such as carageenan and guar gum. Vegetarian replacements for gelatine are not suitable for this investigation as they are made of polysaccharides, not protein.

