Identification of amino acids by chromatography by

Introduction

Chromatography is a common technique used by biochemists in separating and identifying different amino acids and helps to reveal the function of cell organelles. Chromatography is particularly approved for its accuracy in distinguishing between each compound, which it does by separating the chemicals according to their Relative Molecular Mass (RMM). The term was introduced in 1906 by Mikhail Tswett and is derived from the Greek words 'chroma´, meaning colour and 'graphein´, meaning to draw. The most popular type of chromatography employs either absorbent paper, or a dried, thin layer of powder on a glass or plastic base.

There are 20 naturally occurring amino acids. The generalised structure of an amino acid is NH2CHRCOOH.

This consists of an amine group (NH2), carboxylic acid group (COOH) and a distinctive R group bonded to the ?-carbon atom. The R group (or 'side chain´) varies in size, shape, charge, hydrogen bonding, capacity and chemical reactivity. The simplest structure is glycine, which has only an extra hydrogen atom in the side chain. Proteins consist of long chains of amino acids which are held together by chemical linkages called peptide bonds

In this experiment, albumen will be used as the chosen protein. Albumen is globular and has a simple structure. Trypsin will be added (the enzyme functioning in the hydrolysis reaction) to the test tube and it will be given a 24 hour incubation at the ideal temperature of 30oC. The trypsin should then have broken up all the albumen into the separate amino acids.

Aim

To separate and identify a mixture of amino acids by paper chromatography.

Method

Cut a piece of chromatography paper to about 25cms in length and place on a clean surface. To avoid contamination, hold the paper at the top and wear plastic gloves throughout the whole experiment. Draw a line in pencil three centimetres from the bottom of the paper and then four marks lightly along the line at four centimetre intervals.

Using a micropipette, take the prepared albumen from the test tube and lightly dot a small amount on each pencil mark

The paper should then by wafted swiftly over a blue flame to speed evaporation. This process should then be repeated 40 times, applying the same amount of albumen to the same area, to build up a concentration. This is necessary for the chromatogram results to be clear and distinct. Always remain standing and be careful with the evaporation process, as it is very easy for the chromatography paper to go up in flames. The chromatogram should then set up.

The solvent at the bottom of the chromatogram should be made up of: 1 part water, 1 part glacial acetic acid and 4 parts butanol. Leave the paper in the chromatogram for 4 hours (or until the solvent has capillaried up to 5mm from the paperclip). Then remove it and hang it up to dry.

Once the paper is dry, spray it with ninhydrin. The ninhydrin should have the effect of bringing the amino acid dots to visibility. However, ninhydrin is carcinogenic (cancerous), so this must be done in a well ventilated and sparsely populated area, to avoid inhalation and injury. Then wave the paper over a blue flame until all traces are visible. The majority of the dots should appear as purple.

Results

Table 1

Amino acid	Three-letter abbreviation	One-letter symbol	Rf value	Concentration of albumen (%)
Glutamic acid	Glu	E	0.30	16.5
Aspartic acid	Asp	D	0.24	9.3
Leucine	Leu	L	0.73	9.2
Serine	Ser	S	0.27	8.2
Phenylalanine	Phe	F	0.68	7.7
Valine	Val	V	0.60	7.1
Isoleucine	Iso	I	0.72	7.0
Alanine	Ala	A	0.38	6.7
Lysine	Lys	K	0.14	6.3
Arginine	Arg	R	0.20	5.7
Methionine	Met	M	0.55	5.2
Threonine	Thr	T	0.35	4.0
Tyrosine	Tyr	Y	0.45	3.7
Proline	Pro	P	0.43	3.6
Glycine	Gly	G	0.26	3.1

The compounds in the albumen spots dissolve into the solvent and are moved up the paper by capillary action. Depending on the properties of each amino acid, each compound moves at a different speed, leaving amino acid dots at various intervals, which can then be identified by measuring the distance between the spot and the pencil line. The relevant front (Rf value) of each amino acid can then be calculated by using the following equation:

$$\text{Rf value} = \frac{\text{Distance moved by compound}}{\text{Distance moved by solvent}}$$

On the chromatography paper, there is a blurred, dark purple area (approximately 3mm in diameter), 4cms from the origin. If I apply it to the Rf equation:

$$\text{Rf value} = \frac{4}{17}$$

I can calculate that the Rf value is O.235 (3d.p.). In comparing this to Table 1, the amino acid can be successfully identified as being Aspartic acid. Aspartic acid has an albumen concentration of 9.3% and therefore that is why it appears so concentrated on the paper.

Discussion

The completed chromatogram shows the solvent length to be 17cms, by a faint purple disjointed line along the end. At the bottom, the four original dots in purple are distinctly visible and above each, a series of varyingly distinct and faded coloured dots. The majority of these are purple, as all amino acids become purple once sprayed with ninhydrin, with the exception of proline, which turns yellow. There are a couple of anomalous markings, which have been identified as fingerprints. The sweat from the skin contaminates the chemicals present, so there are a couple of readings missing in the fourth line. Some of the readings have also run into each other and are very blurred. In some places the concentration of purple remains constant for as long as 5cms, making it hard to see where each amino acid reading is and so impossible to be fully accurate on the identification.

To check the accuracy of the results, the results can be referred to Table 1. On the chromatogram, a distinct purple mark is visible at 2.5cms. If I put this result in the Rf equation:

$$\text{Rf value} = \frac{\text{Distance moved by compound}}{\text{Distance moved by solvent}}$$

The Rf value of this mark can be found.

$$\text{Rf value} = \frac{2.5}{17}$$

The Rf value turns out to be 0.147(3d.p.), which on the table is most comparable to lysine at 0.14. There is a slight difference in the values; the mark should be at

2.38, for a perfect O.14 value, but in this experiment, the result is sufficiently accurate.
I expected to see a particularly distinct mark for glutamic acid, which has an albumen concentration of 16.5% and is therefore the most prevalent amino acid there. The Rf value of glutamic acid is 0.3, so if the Rf equation is rearranged to:
Distance moved by compound = Distance moved by solvent ? Relevant Front value
Then the area where the glutamic acid spot should have reached can be calculated by multiplying 17 ? 0.3.
The mark made by the amino acid should consequently be visible at around 5.1cms. On my faulty chromatogram, however, there is no visible mark in a 0.75cm radius of 5cms, so I must conclude that my experiment was imperfect and partially inaccurate. The absence of distinct marks from 4.35cms to 5.85cms means that any Rf values from:
4.35 = 0.26(2d.p.)
17
to
5.85 = 0.34 (2d.p.)
17
This also rules out serine (Rf value 0.27) and glycine (Rf value 0.26) as visible dots.
Discussion
The inaccuracy and imperceptibility of many of the results could be due to a number of factors. Firstly, the lack of clarity could be due to the proteins in the albumen solution not being fully broken down. There still may be some peptide bonds holding the amino acids together which would mean that once put in the butanol-acetic acid-water solvent, the proteins would extract water from the solvent, thus causing 'tailing´ on the paper. This could be rectified by leaving the trypsin enzyme for longer in the albumen to complete the hydrolysis, by checking for possible contamination in the trypsin or albumen, or by adding more water to the solvent.
Another possible reason for the inaccuracy could be down to any extraneous chemicals polluting the results. This could be sweat from fingertips, where flawed or no plastic gloves were worn; or foreign chemicals from the micropipette. It is unlikely the pipette was contaminated however, as they were made especially for the experiment and had been sterilised by the flame. It is also improbable that the chemicals used in the solvent were polluted, but the butanol (a form of alcohol) may have affected the proteins and caused a reaction.
The chromatogram should have been left for four hours, instead of which, my experiment was only left for 2 ½ hours, so there may not have been sufficient time for the compounds to properly dissolve in the solvent. A longer amount of time would enable the paper to be left in for the allocated time. If I had been given longer time on this experiment, I would also have been able to build up a stronger concentration of the trypsin/amino acid mixture on the paper. Instead of 40, I was only able to build up the concentration 8 times, which is one fifth of the intended amount. This probably affected the clarity and strength of colour in the dots and explains one reason for the faded pale marks in some areas of the chromatography paper.
The only mark that did not turn purple once sprayed with ninhydrin, is proline. Proline is individual in that it is bonded to both the nitrogen and the ? -carbon atoms. The resulting cyclic structure influences the protein architecture and means that it turns yellow. It is called an imino acid and belongs to the secondary amino group. Because of its unique colour it is the normally the easiest to find on the paper. If I calculate the distance moved by the compound by the Rf value (0.43) and the distance moved by the solution (17cms), the mark should be observable at 7.3cms (1d.p.). On the chromatogram, I can see a yellow mark at 7.4cms, which is inaccurate by 0.1cms, but this is not disastrous.

Discussion
There are other methods of chromatography that I would be interested in investigating. In 2D chromatography there is limited 'tailing´ in the results, which is an improvement to the previous method. By using 2 different solvents in the sequence this technique gives better separation compared with 1-Dimensional chromatography. The molecules are given 2 'runs´ so any errors made in the blurring of the first run do not affect the results in the second run.
Another method in chromatography is electrophoresis. In electrophoresis, the proteins used are placed on a moist filter paper or gel and put in an electric field Each moves a characteristic distance on the paper/gel and when dried, the amino acids can be identified similarly to that of paper chromatography. The charged substances move towards the opposite end and the negligibly charged substances are separated by forming ionic bonds with ions present in the solution. The charge of the protein molecule depends on the amino acid composition and the pH of the solution and so can be separated by charge. This is a good method and would be a possible future method as the apparatus and method aren´t very complex.

Discussion (page 4)
One of the problems experienced using paper chromatography is that the fibres vary in size and shape, which naturally limits the precision and means that the spots are less resolute. In the Edman Degradation, the amino acids in the hydrolysates can be separated by ion exchange chromatography on a sulfonated polystyrene resin (eg Dowex-50). They can then be quantitated by reacting them with ninhydrin. ?-Amino acids treated with this method turn an intense blue shade, whereas imino acids (eg proline) turn yellow. The concentration of the amino acids in the solution is proportional to the optical absorbency of the solution after treating it with ninhydrin. This technique is a very valid one as it is very sensitive and can detect a microgram (about 10nmol) of an amino acid.
In Thin Layer Chromatography, sephadex gel is usually used which increases the speed of the experiment by up to 12 times. It is more accurate than paper chromatography, as the gel beads are uniform and give more distinct and precise dots, with minimal blurring. This would be an excellent alternative for a future investigation.
Column chromatography is very popular with professional biochemists. There are problems in obtaining the right packing material for the experiment, as it must allow constant packing and unobstructed emanation of the solvent through it. The solvent must also be uncontaminated and pure to ensure a proper development. They must be added in order of the least to the most polar (eg water). Then the fraction is reacted with an appropriate agent and identified.
A partition column is used similarly and can be compared to fractional distillation. It is not ideal, as the separated components would be too small and indistinguishable. It is also not as speedy as Thin Layer Chromatography, or as simple a method as electrophoresis. In the method, the amino acids are separated over time (according to each composition) into bands and chromatography paper is then applied to check the purity. This step is good as it checks for contamination, which paper chromatography does not.
Final Conclusion and Evaluation
The results on my chromatography paper are largely indistinct, with a couple of exceptions, so there are too many anomalies to make a complete accurate assessment of the identification. However, it has given me a fair estimation of the ideal results and so has not been entirely unsuccessful.
In the investigation I encountered many problems and made a few careless errors in what should have been a simple experiment. If I was repeating this investigation, I would like more time, which I think would improve the accuracy of the results.
? I would leave the albumen/trypsin solution in for 28 hours in a sterile

environment to ensure that the protein had been fully broken down into the amino acids and that there had been no contamination.
? I would make sure that I was wearing a strong pair of plastic gloves at all times and that I was only holding the top of the paper where the paperclips were inserted. This would avoid the problem of irrelevant chemicals contaminating the results.
? The utmost care and attention would be sent on evaporating the albumen in the blue flame. In the investigation, the first piece of chromatography paper was held over the flame for a little too long and it burnt.
? Time would be spent guaranteeing that the albumen spots were built up a minimum of 40 times, to ensure a adequate concentration of the amino acids.
A few adjustments would be made to the method as well.
? There is a risk of peptide bonds that are still unbroken in the chromatogram. This can lead to the proteins extracting too much water from the solvent, causing 'tailing´. In a future experiment, a greater concentration of water would be added to the solvent to minimalise any blurring of the results.
? All equipment used in the investigation would be sterilised with boiling water prior to the experiment. This would reduce the number of anomalous readings on the paper.
Nevertheless, if I was repeating the paper chromatography investigation I would keep some constants.
? I would keep trypsin as the enzyme as it is ideal for a large yield on digestion with protein. It also acts quite quickly and reliably.
? I would probably keep using albumen as is a simple polypeptide and so not too difficult to break down.
If the investigation was being repeated but with a different chromatography method, I would like to carry it out using Thin Layer Chromatography. The method appears to be simple and much more concise than paper chromatography. It is also a lot quicker and can be executed in as little as 20 minutes. This would mean that I would have 35 minutes in which to carry out the experiment carefully and concisely.