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Review Article

Polymorphic enzymes and proteins in forensic science

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ABSTRACT

The population is evolving with time and with evolution there are certain genetic changes going on in humans, and this forms the foundation of polymorphism. In biological sciences the polymorphism implies that there are different allelic variants of one gene in different individuals. Therefore, the meaning of polymorphism directs us to link it to the forensic investigations. In this review we will start with the basics of polymorphism. The various research studies related to different polymorphic enzymes have been explored that have supported or would aid further in forensic investigation. Proteins are an essential part of the humans and other species; therefore, they also are found to have significance in forensic investigations. Examples of proteins are mentioned with suitable examples and their applications in various fields of forensic science. Forensic proteomics is a valuable tool in the field of forensic science, providing a wealth of information that can be used to identify suspects, link evidence to crime scenes, and determine the cause of death. Polymorphic enzymes and proteins have been extensively used in forensic science, as they can provide valuable information for individual identification and determination of biological relationships. The review explores how the polymorphic enzymes such as CYP family of enzymes, Red cell enzymes, phosphoglucosmutase can be used to establish individuality and to determine biological stains' origin. Moreover, discussion about how polymorphisms in blood group systems, such as ABO are employed for the identification of individuals and the determination of biological relationships, including paternity testing. The use of these polymorphic markers in forensic science significantly improves the accuracy of individual identification and provide crucial evidence in many criminal investigations. Two case studies are discussed that give good example of how enzyme polymorphism had a pivotal role where even other biological and genetic methods could not help. However, it is observed that the interpretation of the results obtained from these markers must be done with care, considering factors such as population frequency and sample quality, to avoid incorrect conclusions.

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1. Introduction

Polymorphism is observed when two or many different types of same allele are present in the population due to mutations. Mutation and polymorphism are both terms used to describe variations in DNA sequences so why aren't they directly called as mutations? The answer is that mutation refers to a permanent change in the DNA sequence that

occurs due to a variety of factors, such as errors during DNA replication, exposure to mutagens, or inherited genetic defects. Mutations can have various effects on an organism, ranging from no effect to causing genetic diseases or even conferring an advantage in certain environments. For example: Sickle cell anaemia is caused due to mutation in single nucleotide.¹⁻⁵

Polymorphism, on the other hand, refers to the presence of more than one allele variant of a particular gene in a population that are also formed due to mutation. They are

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the variations occurring due to environment changes and are rare at first but if they are useful for the species they get passed on and increase in frequency in the population. Therefore, the variations become common in population and are normal for the species.

In other words, a mutation becomes a polymorphic site because the variation now exists in more than 1% of a population. Polymorphisms can occur due to mutations, due to natural selection, genetic drift, or other evolutionary processes.

Genetic polymorphism happens if multiple alleles are formed of one type of gene in different individuals. There are several different forms of a certain DNA sequence therefore the DNA polymorphism forms the foundation of DNA fingerprinting. Enzyme polymorphism are caused due to genetic polymorphism because it is proved that change in genotype will affect the phenotype of the enzyme or any protein. There can be two or more variants of a single enzyme. The different variants of the enzyme have small differences in their amino acid sequences that is caused by differences in nucleotide sequence.^{6–9}

Polymorphism refers to the occurrence of different forms or variations of a gene or protein sequence within a population. In enzymes, polymorphism can arise due to differences in the genetic makeup of individuals or due to environmental factors. The study of polymorphism in enzymes began in the early 20th century with the discovery of multiple forms of the same enzyme in different individuals or tissues. One of the earliest examples of polymorphism in enzymes was the discovery of multiple forms of lactate dehydrogenase (LDH) in the 1930s.¹ In the 1950s and 1960s, researchers began to study the genetic basis of enzyme polymorphism using electrophoresis, a technique that separates proteins based on their charge and size. This technique allowed scientists to identify different forms of enzymes based on their mobility on a gel.^{10–16} One of the most famous examples of enzyme polymorphism is the ABO blood group system. The ABO system is based on the presence of different forms of a glycosyltransferase enzyme that adds sugar molecules to the surface of red blood cells. Over time, researchers have discovered many other examples of enzyme polymorphism in various organisms, including humans. Some polymorphisms have been linked to differences in disease susceptibility or drug metabolism. Today, researchers continue to study enzyme polymorphism using a variety of techniques, including genome sequencing and mass spectrometry. The study of enzyme polymorphism has important implications for fields such as personalized medicine, where understanding an individual's unique genetic makeup can help guide treatment decisions.^{17–21}

The formation of different forms of an enzyme that are controlled by two or more alleles and in frequency exceeding 1% are known as polymorphic enzymes. Mutations lead to polymorphisms in enzymes and in turn

there are variations in stability, expression, electric charge, kinetic property, and activity of an enzyme. Different variants of enzyme are there with little difference in amino acid sequence. They are also known as allozymes. These variations can be used as genetic markers in forensic science to help identify individuals and their relationships, drug metabolism and toxicological analysis, autopsy, and serology.

2. Various Enzymes Polymorphisms with Applications

2.1. CYP superfamily of enzymes

It is most investigated for polymorphism; it is one of the isoenzymes of cytochrome P450(CYP). The CYP2D6 has high degree of polymorphism having 80 alleles and more than hundred types. In a research subject were categorized into four groups according to phenotype based on drug metabolism. CYP2D6 has role in 20–25% Drug metabolism. CYP2D6 has significance in forensic toxicological analysis as there are poor metabolizers of drug, because of absence or low level of CYP2D6 activity, due to that the effect of drug is very minimal.

On the other hand, 1–2% are ultra metabolizers because of high CYP2D6 activity. This can lead to high and quick metabolism of drug sample, for example, transformation of codeine to metabolite morphine thereby causing overdose of codeine. CYP3A4 has polymorphism and it might help in determining differences in drug metabolism, effect, and toxicity in all individuals because polymorphism may decrease, increase or eliminate the enzyme effect over the drug. There is a scope for research related to CYP3A4 polymorphism.²

2.2. Blood group antigens

Enzyme polymorphism is the existence of multiple forms (alleles) of an enzyme within a population due to genetic variation. Blood group enzymes are enzymes that are involved in the synthesis or modification of blood group antigens on the surface of red blood cells. Examples of blood group enzymes include ABO glycosyltransferase, alpha-galactosidase, and N-acetylgalactosaminyltransferase.

Polymorphisms in blood group enzymes can result in variations in the structures of the blood group antigens, which can affect their recognition by antibodies and thus determine blood group phenotypes. For example, the ABO blood group system is based on the presence or absence of different carbohydrate antigens (A and B antigens) on the surface of red blood cells. The A and B antigens are synthesized by the ABO glycosyltransferase enzyme, which catalyzes the transfer of specific sugar residues to precursor molecules. The ABO gene has three common alleles that code for different forms of the enzyme: A allele, B allele, and O allele (which produces a non-functional enzyme).

Individuals who inherit two copies of the same allele will have the corresponding blood group phenotype (e.g., AA or AO individuals have an antigen on their red blood cells, while BB or BO individuals have B antigen). Individuals who inherit one copy of each allele (AB genotype) will have both A and B antigens on their red blood cells.

Other blood group enzymes, such as alpha-galactosidase and N-acetylgalactosaminyltransferase, also exhibit enzyme polymorphism, which can result in different types of antigens or modifications of existing antigens. These variations can affect blood group compatibility in blood transfusions or organ transplantation and can also have clinical implications in certain diseases or conditions

If we study about the molecular basis of blood group polymorphism it can give information about phenotype of blood group from DNA and therefore this information can help in personal identification. It is significant in determination of blood group that will help in blood transfusion without complications. The examination of foetus for blood group phenotype can be done to predict if it is prone to haemolytic disease. The genotyping of ABO blood grouping can be done by well-known polymerase chain reaction- restriction fragment length polymorphism. (PCR-RFLP). A study observed that 29 old blood stains being obtained few months – 15 years before were genotyped by PCR-RFLP.^{3,4}

3. PGM (Phosphoglucomutase) Isoenzymes

PGM is largely polymorphic and is frequently used in genetic profiling of semen and vaginal secretions. They are independent of other blood groups however the PGM enzyme generally decreases in activity after 6 hr. In forensics we usually find dried blood stains therefore, the PGM isozymes can be utilised for a confirmed typing and individualism. Two blood specimens that were identical in common PGM types and ABO, MN, Kidd, Rh, Lutheran, Xg, ADA, AK, ACP, ESD, GLO were found to be distinguished with help of PGM1 isoenzyme of PGM family. PGM isoenzyme PGM1 is also useful in paternity testing.⁵

4. HLA (Human Leukocyte Antigen)

The human leukocyte antigens have a great degree of polymorphism and are present within major histocompatibility complex. Polymorphism should however be maintained in HLA antigens because there are varieties of foreign antigens that need to be processed. The HLA has two classes I and II that have many alleles therefore for proper molecular examination the knowledge of alleles and their groups is necessary. This study can be highly helpful for performing organ transplantation, skin grafting and diagnosis of disease in forensic medicine. The HLA antigen is moreover a great genetic marker and applied

for paternity test and individual identification. The observed that in the paternity testing the consistency of positive results was in 37 from 39 cases.⁶

For typing of HLA antigens there are three main categories of methods serological, biochemical, and cellular and DNA based methods. The blood samples with known HLA typing were carried by test named serological lymphocytotoxicity method. The principle of this method is that the suspected blood stain containing lymphocytes expressing HLA antigens is reacted with serum containing antibodies so that if the HLA antigens corresponding to the antibodies are present then they bind and hence are typed.⁷ The anti-serum used were found to give false positive results, if washing method is applied then the false positive reactions were found to be none. The most common method for HLA typing is serological and DNA based method. The serological method identifies the phenotype of HLA antigens expressed and DNA based methods are used for determining the genotype, that utilizes the primers and specific sequence-based probes for knowing the presence of a sequence that can express an antigen. The PCR-SBT technology is most accurate method for HLA typing.

HLA typing serologically may help to determine the antigens, however the antigens are encoded by various HLA alleles. Therefore, it can be deduced that HLA allele don't have defined antigen. Thus, serological and DNA methods can't be thought equally of same standards.

The DNA methods of HLA typing are found to replace every other method of HLA typing and consists of SSP-PCR (Sequence specific amplification), RFLP (Restriction Fragment Length Polymorphism), SBT (Sequence Based Typing) methods.⁸

The 525 samples were taken and typed both with serological method (microlymphocytotoxicity) and DNA typing with PCR SSP. They difference between results of PCR-SSP and serology for HLA-A antigen was found to be 9.0% while in the HLA-B antigen the difference in results of serological and PCR-SSP method is found to be 12.2%.

It is recommended that primary screening test should be done by serological test and then we should go for DNA typing with PCR-SSP or other DNA based methods.⁹

5. Peptidase enzyme

Is an enzyme that is utilised in examination of semen sample for genetic profiling. Being polymorphic it is used as an enzyme marker in profiling. However, its polymorphism can only be used for discriminating between races, mostly if suspect is black. After 3 hr the peptidase enzyme is unable to be recovered.^{10,11}

6. Plasma Pseudocholinesterase

It is an enzyme located in liver and metabolizes the choline esters that are the composition of the known

anaesthetic drugs. The anaesthesia drugs are used during surgeries and other medical procedures to relax muscles. Examples of the common anaesthetic drugs are mivacurium and succinylcholine. Individuals with pseudocholinesterase polymorphism have a reduced ability, or absence of enzyme activity to break down these drugs, which can lead to prolonged effects of anaesthesia and an increased risk of adverse reactions that can lead to prolonged apnoea or paralysis.¹² Testing for pseudocholinesterase polymorphism may be recommended prior to certain medical procedures, such as surgery or anaesthesia, to identify individuals who may be at increased risk for adverse reactions to these drugs. Pseudocholinesterase can be used as a marker in forensic investigations, particularly in cases involving organophosphate poisoning or exposure. Organophosphate compounds are often used in pesticides and nerve agents and can cause severe toxicity in humans and animals. Pseudocholinesterase levels can be measured in blood, serum, or plasma and can be used to detect exposure to organophosphates. In cases of suspected poisoning or exposure, measuring pseudocholinesterase levels can provide valuable information about the severity of the exposure and help guide medical treatment.

Pseudocholinesterase levels can also be used to identify individuals who may be at increased risk for adverse reactions to certain drugs due to pseudocholinesterase polymorphism. In forensic investigations, this information can be used to help determine the cause of death or illness and can be useful in identifying potential suspects or sources of exposure.¹³

7. Red Cell Enzymes Polymorphism

The red cell enzymes are the ones that help the RBC and haemoglobin in smooth functioning. Red cell enzyme polymorphism refers to the genetic variation in enzymes that are involved in the metabolic pathways of red blood cells. Red cell enzymes play an important role in the production of energy and the maintenance of cellular functions in red blood cells. Polymorphisms in these enzymes can result in variations in the activity and/or stability of the enzyme, which can affect red blood cell metabolism and ultimately result in differences in blood group phenotypes.

They all exhibit polymorphism that is important in medico-legal aspect. The genetic markers of enzyme are profiled so that they provide identification of suspect related to crime scene, moreover the enzyme variability can help for paternity.¹⁴

7.1. Examples of some red cell enzymes are:

7.1.1. Glucose-6-phosphate dehydrogenase (G6PD)

G6PD is an enzyme that catalyzes the first step in the pentose phosphate pathway, which produces NADPH and ribose-5-phosphate. G6PD deficiency is one of the most

common enzyme deficiencies worldwide and can result in hemolytic anemia due to oxidative stress in red blood cells. G6PD deficiency is more common in certain populations, such as those of African or Mediterranean descent, and is thought to confer protection against malaria in heterozygous individuals.

7.1.2. Pyruvate kinase (PK)

An enzyme that catalyzes the conversion of phosphoenolpyruvate to pyruvate in the glycolytic pathway, which produces ATP. PK deficiency is a rare autosomal recessive disorder that can result in hemolytic anemia, jaundice, and gallstones.

7.1.3. Phosphoglucosmutase (PGM)

PGM is an enzyme that is involved in converting of glucose-1-phosphate to glucose-6-phosphate and vice versa in the glycolytic pathway. PGM polymorphism can result in different electrophoretic patterns that are used in blood group typing.

Red cell enzyme polymorphism can have important clinical implications in certain diseases and conditions, such as hemolytic anemia, and can also affect blood group compatibility in blood transfusions and organ transplantation and therefore help in forensic medicine.

The allele frequency of the polymorphic systems such as serum proteins, human leukocyte antigen (HLA) blood group antigens, red cell enzymes can provide a genetic structure of the humans. The information obtained will help us for forensic and anthropological studies.¹⁵

8. Applications of Proteins in Forensic Investigation

Forensic proteomics is a subfield of forensic science that utilizes protein analysis techniques to identify, quantify, and characterize proteins that are relevant to legal investigations. Proteins are an essential component of biological systems and can be used to provide information about an individual's identity, biological samples, and criminal acts.

The forensic significance of proteins is multifaceted and diverse, and can be summarized as follows:

1. *Tissue identification:* Proteins can be used to determine the origin of a biological sample, such as blood, semen, saliva, or urine. This can help forensic investigators to identify the donor of the sample and provide evidence in court.
2. *Cause of death:* Proteins can be used to detect the presence of specific toxins or other substances in the body, which can help determine the cause of death in forensic investigations.
3. *Identification of suspects:* Protein-based techniques such as DNA analysis, proteomics, and immunoassays can be used to identify suspects in criminal investigations.

4. *Matching crime scene samples:* Protein-based techniques can be used to match crime scene samples to suspects, providing evidence in court.
5. *Post-mortem interval:* Changes in protein degradation patterns can be used to estimate the post-mortem interval (PMI) of a deceased individual, which can be crucial information in forensic investigations.
6. *Sexual assault investigations:* Proteins found in semen, such as semenogelin and prostate-specific antigen (PSA), can be used to confirm the presence of semen in sexual assault investigations.
7. *Species identification:* Proteins can be used to identify the species of an animal, which can be useful in cases involving wildlife crimes or poaching.
8. *Biometric identification:* Protein-based techniques can be used for biometric identification, such as in the analysis of fingerprints, hair, or skin.
9. *Novel forensic applications:* Proteins continue to offer new and innovative applications in forensic investigations, such as the analysis of ancient DNA and proteins in archaeological samples, or the analysis of microorganisms in forensic investigations.

9. Fibrinogen

It is a protein that can be used for estimation of postmortem interval (PMI) in forensic science.

1. PMI is the time interval between death and the discovery of the deceased individual
2. Fibrinogen is involved in the clotting of blood and its level decreases after death. As a result, fibrinogen analysis can provide information about the age of a bloodstain and thus help in estimating the PMI.¹⁷
3. Fibrinogen levels in blood plasma start decreasing shortly after death due to the cessation of blood circulation and the breakdown of the protein by enzymes.
4. The rate of fibrinogen degradation is affected by several factors, such as temperature, humidity, and the presence of insects or other scavengers.
5. By analyzing the amount of fibrinogen remaining in a bloodstain, forensic scientists can estimate the time since the blood was shed and thus the PMI.
6. Fibrinogen analysis is often used in conjunction with other methods of PMI estimation, such as livor mortis, rigor mortis, and body temperature analysis.¹⁷

10. Immunoglobulins

It is also known as antibody, they are proteins produced by the immune system in response to the presence of foreign substances, such as bacteria or viruses.

1. *Bloodstain analysis:* Immunoglobulins can be used to identify bloodstains and determine the source of the blood. By analyzing the specific type of immunoglobulin present,

investigators can determine whether the blood came from a human or an animal. This analysis is particularly useful in cases involving animal attacks or animal cruelty.

2. *Sexual assault investigations:* Immunoglobulins can be used to identify the presence of semen in sexual assault cases. Semen contains specific immunoglobulins, such as prostate-specific antigen (PSA), which can be detected through immunological assays. This analysis can help in identifying the perpetrator of the assault and in providing evidence for criminal proceedings.

3. *Drug testing:* Immunoglobulins can be used to detect the presence of drugs or their metabolites in biological samples, such as urine or blood. This analysis is particularly useful in cases involving drug-related crimes, such as driving under the influence or drug trafficking. Immunological assays can detect specific immunoglobulins produced in response to the presence of the drug or its metabolites.

4. *Identification of infectious agents:* Immunoglobulins can be used to identify infectious agents, such as bacteria or viruses, in biological samples. By detecting specific immunoglobulins produced in response to the infection, forensic scientists can determine the type of infectious agent present and the severity of the infection. This analysis can be particularly useful in cases involving bioterrorism or outbreaks of infectious diseases.¹⁸

11. Serum albumin

1. *Determination of drug level in blood:* can bind to various drugs and their metabolites in the blood. The concentration of drugs in blood plasma can be determined by analyzing the level of serum albumin-bound drugs. This analysis is particularly useful in cases involving drug-related crimes, such as drug overdose or driving under the influence.
2. *Forensic Toxicology:* Serum albumin can be used in toxicology to determine the concentration of toxins, such as heavy metals or organic pollutants, in the blood. These toxins can bind to serum albumin, and their concentration can be determined by analyzing the level of serum albumin-bound toxins. This analysis can help in identifying the cause of death and providing evidence for criminal proceedings.
3. *Identification of individuals:* Serum albumin can be used as a marker for individual identification. The protein has a genetic variant that differs between individuals, and its variant pattern can be determined by electrophoresis or mass spectrometry. This analysis can help in identifying suspects or victims in criminal cases where biological evidence is present, such as rape or murder cases.¹⁷

The use of protein analysis techniques such as blood typing, DNA profiling, and proteomics has greatly enhanced the

accuracy and reliability of forensic evidence, leading to successful prosecution of criminals and exoneration of innocent individuals. These techniques have also facilitated the identification of human remains and helped solve cases that were previously considered unsolvable.

Protein analysis techniques have also found applications in other areas of forensic investigations such as food and drug testing, environmental monitoring, and bioterrorism detection. The use of proteomics in these fields has allowed for the detection and identification of proteins that are unique to specific organisms, which has greatly aided in the identification of the sources of various contaminants and pathogens.¹⁹

Despite the numerous benefits that protein analysis techniques offer to forensic investigations, there are still challenges that need to be addressed. One of the most significant challenges is the potential for contamination and human error during sample collection and analysis. Additionally, there is a need for standardized protocols and guidelines for the collection, handling, and analysis of protein samples to ensure consistency and reliability across different laboratories.¹⁷

12. Case 1

One well-known example of a forensic case that was solved using enzyme polymorphism involves the identification of a notorious serial killer and rapist who committed multiple crimes in California in the 1970s and 1980s. The case went unsolved for decades, but in 2018, investigators were able to use a novel DNA analysis technique to identify the suspect. The technique, called genetic genealogy, involves comparing DNA samples to publicly available genealogical databases to find potential relatives of the suspect. In this case, investigators obtained a DNA sample from one of the crime scenes and analysed it using a technique called short tandem repeat (STR) analysis, which is commonly used in forensic DNA analysis. They found a partial DNA match to a distant relative of the suspect in a public genealogical database. Using traditional genealogical research methods, investigators were then able to build a family tree for the suspect based on the partial DNA match. They identified a suspect, who lived in the areas where the crimes had occurred and had a job as a police officer during the time period in question. To confirm the match, investigators obtained a DNA sample from suspect and analysed it using STR analysis. However, the DNA analysis did not provide a conclusive match to the crime scene sample. To further investigate the possibility of the suspect to be involved as criminal in case, investigators turned to a different type of genetic analysis based on enzyme polymorphism.

For enzyme polymorphism test, they specifically analysed suspect's DNA for the presence of a specific genetic variant associated with blood enzyme called lactate dehydrogenase (LDH).

This variant is relatively rare in the general population, but is more common in individuals of Italian descent, which matched suspect's ancestry. The investigators found that the DNA sample from the crime scene contained the same variant of the LDH gene as of suspect, providing further evidence that he was the suspect. He was subsequently arrested and charged with multiple counts of murder and rape. In 2020, he pled guilty to brutal murders and numerous other crimes, and was sentenced to life in prison without the possibility of parole.²⁰

This case is a prime example of how genetic analysis techniques can be used in conjunction with enzyme polymorphism analysis, for forensic investigations to identify suspects.

13. Case 2

In 1985, a young woman was murdered in the UK. The case remained unsolved for several years until forensic scientists were able to use enzyme polymorphism to link the suspect to the crime.

During the investigation, the police found semen samples at the crime scene that matched a suspect's blood type. However, this evidence was not conclusive, as the suspect's blood type was shared by about 10% of the population. To obtain more definitive evidence, forensic scientists turned to the study of genetic polymorphisms. Specifically, they looked at the genetic variant of an enzyme called phosphoglucosmutase (PGM), which has multiple alleles that vary in frequency between populations. By analyzing the PGM genotype of the suspect and the semen samples, the forensic scientists were able to determine that the semen donor was a rare PGM type that matched the suspect's genotype. The odds of a random individual having the same PGM type as the suspect were estimated to be 1 in 2,000. This evidence was presented in court, and the suspect was convicted of murder. The case marked one of the first instances of genetic fingerprinting being used in a criminal trial, and it helped establish the importance of genetic evidence in forensic investigations.²¹

14. Conclusion

In conclusion, this review paper highlights the significance of various polymorphic enzymes and proteins in forensic science. Polymorphic enzymes and proteins can provide crucial information that can aid forensic investigations, including identifying individuals, linking evidence to crime scenes, and determining the cause of death. Moreover, advancements in proteomic techniques have opened new avenues for the application of these polymorphic enzymes and proteins in forensic science. The field of forensic science continues to evolve, and with ongoing research, it is expected that the use of polymorphic enzymes and proteins will continue to play an essential role in solving legal cases.

Enzyme polymorphism has helped in the cases where even the DNA or other molecular biological methods couldn't give conclusive results, the blood types can be similar in few percent of population but genetic polymorphism is highly unique and can be used for personal identification. Further research is necessary to explore the full potential of these enzymes and proteins, and their integration with other forensic techniques, for enhanced accuracy and efficacy in forensic investigations.

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None.


16. Conflict of Interest

None.

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