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Abstract

Amylase was the first enzyme to be characterised, and for the past 200 years, its clinical role was restricted to a diagnostic aid. Recent interface research has led to a substantial expansion of its role into novel, viable diagnostic and therapeutic applications to cancer, infection, and wound healing. This review provides a concise “state of the art” overview of the genetics, structure, distribution, and localisation of amylase in humans. To the four classical hyperamylasemia patterns (pancreatic, salivary, macroamylasemia, and combinations) a fifth, the localised targeting of amylase to specific foci of inflammation is proposed. The implications are directed at novel therapeutic and diagnostic clinical applications of amylase such as the novel therapeutic drug classes capable of targeted delivery and “smart release” in areas of clinical need. Future directions of research in areas of high clinical benefit are reported.

1. Introduction

Infection, cancer impaired wound healing continue to present formidable challenges to the current practice of surgery.[1-4] Combined, these represent the main cause of mortality, morbidity and recurrent health expenditure worldwide.[5] Additionally, the disproportionate increase in multidrug resistant infection against the decline in development of novel antibiotics has been recognised as a global threat in many areas of medicine and surgery.[6-8] Therefore, novel developments with the potential to improve diagnostics and therapeutics across all three fields are highly sought after.

Amylase was the first enzyme to be discovered and isolated by Payen in 1833.[9, 10] Over the last 190 years, its use in the diagnosis of pancreatic and associated diseases has been well-established.[11] The last 20 years have witnessed intensive research into expanding the clinical applications of this venerable enzyme with high relevance to surgical management, including the treatment of surgical infection, solid cancer, and wound management. This study provides a concise "state of the art" overview of advances in basic science concerning amylase structure, function, and bio-distribution, leading into clinically relevant diagnostic and therapeutic applications that have reached clinical practice or advanced stages of development. The review draws on advances at the interface of surgery, diagnostics, synthetic chemistry, and nanomedicine to indicate clinically valid directions for future research.

2. Methods

A first generation literature search was performed using the MeSH [12] search string "Amylase AND (diagnost* OR therapeut$)" on the OVID-SP and PUBMED platforms. 1755 studies were retrieved and screened for relevance. A second generation search was then performed by forward and backward referencing on Web of Knowledge™ and manual indexing. Only articles with full text available in English were included.

3. Literature Review

3.1 Amylase Enzyme Classification

Amylases are part of a large group of glycosyl hydrolase enzymes that catalyse the breakdown of complex carbohydrates.[13, 14] Fifty seven families of glycosyl hydrolases have been classified based on their function and specificity [15] (Table 1). Of the three main classes of amylase enzymes (α, β and ɣ), α-amylase is found in animals, plants, fungi, and bacteria, β amylase is found in plant seeds, bacteria and fungi and ɣ amylase is found in yeast and fungi. The main function of all of the amylases is to degrade starch and sugars. However, there is a difference in their structure and mode of action. α-amylase cleaves randomly along the starch chain and because of this non-specificity, digestion is more rapid than other amylases.[16]

3.2 Human α-amylase: genetics and function-structure considerations

Salivary α-amylase is expressed in salivary, mammary, and lacrimal glands, while the pancreatic isoform is only expressed in the pancreas.[17] Pancreatic α-amylase constitutes 5-6% of the total protein present in pancreatic secretions, and unlike many other pancreatic enzymes, it has no inactive precursor.[18] Gene structure suggests that the human salivary amylase gene has evolved from a pre-existing pancreatic amylase gene. Figure 1 reports the relationship between structure, function, and genetics of this enzyme family.

3.3 Mode of action, cofactors, and clinically relevant substrates

Current assays only measure activity, and therefore the mode of action, environmental conditions, presence of cofactors, and substrate nature merit consideration. Figure 2 illustrates that the mode of action of α-amylase is a double displacement mechanism. Human α-amylase requires two essential

cofactors for its activity: chloride and calcium ions.[19] The α-amylases are calcium metalloenzymes and are unable to function in the absence of calcium.[19] The calcium ion is coordinated between 8 ligands from the main amino acid chain, side chains, and three water molecules while the chloride ion binds closely to the active site and is considered to act as an allosteric activator. Several medical devices chelate calcium, possibly affecting amylase activity, for instance alginate dressings.[20, 21] Several amylase inhibitors have been identified and used clinically in the treatment of human disease (Section 3.7). Whereas the physiologic pH range of blood is 7.35-7.45, the optimum pH for amylase activity is 6.7 to 7.0, which would be expected in pathophysiologic states such as infection and possibly solid tumors.[22] Localised amylase concentrations are increasingly important in the development of amylase-based therapeutics (section 3.5). Such devices and medical dressings may also adversely affect the amylase assays in current use because they are activity, not concentration based (section 3.5).

3.4 Distribution in Pathophysiologic States

The classic diagnostic use of α-amylase presupposes that the plasma concentration of α-amylase reflects the pathophysiologic processes in the source organ, and increases in α-amylase activity in human serum has traditionally formed the basis of its clinical applicability. Four patterns of generalised hyperamylasemia have been recognized: pancreatic, salivary, macroamylasemia, and combinations thereof.[19] Pancreatic and salivary disease are described extensively elsewhere.[23] Macroamylasemia is the increase of serum α-amylase due to a macromolecular complex between amylase and immunoglobulins which imparts increased size and molecular weight. This benign biochemical aberration only affects approximately 0.4% of the general population.[24-26] but can be a source of diagnostic confusion.[27] Table 2 provides examples of clinical conditions associated with hyperamylasaemia. Additionally it is proposed that amylase can sequestrate locally at operative sites of enhanced vascular permeability or bacterial infection.

3.4.1 The Enhanced Permeability and Retention (EPR) Phenomenon

The EPR effect is mediated by the enhanced vascular permeability and distorted/dysfunctional lymphatic drainage of macromolecules at sites of localized inflammation.[28] Large molecules such as amylase may therefore be retained in areas of enhanced vascular permeability and distorted drainage such as infection. It has been recently proposed that the EPR effect is mediated by Bradykinin, resulting in localised hyperpermeability of the microvasculature. Based on this notion, novel bio responsive antibiotics have been described recently.[2, 29, 30] Evidence of localisation of amylase to surgical site infection has been claimed from a consecutive series of patients (n=6) with infected burn wounds,[31, 32] but it is known that amylase is present in sweat. Interestingly, histamine has also been reported recently to stimulate secretion of pancreatic α-amylase into the gastrointestinal tract and increase vascular permeability.[33-35]

3.4.2 Bacterial amylase

Bacterial amylase is essential to the *Bacillus* and *Clostridium* genus.[36-40] Such bacteria cannot absorb starches and therefore, α-amylase is constitutively secreted, digesting glucose which can then be absorbed.[41] Interestingly, organisms in this genus are capable of prolific amylase secretion, and may consequently increase amylase levels around the infected site by several orders of magnitude.[41-47] Several organisms in these genuses are responsible for life-threatening human infection, often requiring urgent treatment including *C. perfringens* (gas gangrene), [48] *C. tetanii* (tetanus), [49] *C. difficile* (hospital acquired pseudomembranous colitis),[50, 51] and *B. cereus* (food poisoning). Several *Bacillus* species are exploited in the alimentary biotechnology industry for their prolific amylase production.[52, 53] Harnessing this phenomenon for clinical diagnostic and / or therapeutic applications, however, has not yet been reported. Additionally, *Aeromonas hydrophila*, a Gram negative pathogen, constitutively expresses

Dextrin and dextran are mentioned here as two salient examples. Dextrin is a α-1, 4 poly (D-glucose) polymer obtained from hydrolysis of starch. Dextrins are largely linear polymers with limited (< 5%) branching in the α-1, 6 position and have an established safety profile.[63, 64] Dextrin's versatility to several clinical applications has been well-established. Dextrin is used as a supplement for renal and hepatic failure [65] as a carrier for intraperitoneal 5-fluorouracil, [66, 67] and as a component of peritoneal dialysis solutions. [68] In contrast, dextran is a complex, branched glucose polymer which consists of both α-1,6 glycosidic linkages and branches at α-1,3 linkages.[69] Dextran is used medicinally as an antithrombotic (antiplatelet) to decrease blood viscosity and as a volume expander in hypovolaemia.[70] Amylase is the enzyme which provides a predictable, safe degradation of these polymers in the bloodstream, eventually resulting in hydrolysis to simple sugars that can be fully metabolised. In this regard, α-amylase triggers hydrolysis of α-1,4 glycosidic linkages forming maltose and iso-maltose which are metabolised subsequently to glucose by tissue maltases, and/or excreted into urine.[71-73] The importance of amylase in this context is often overlooked. Consequently, the effect of co-incidental disease that affects amylase levels, which may dictate the duration of the intended therapeutic effect, is often overlooked (Table 2).

An extra level of complexity can be achieved if these polymers are combined chemically to bioactive, conventional molecules of interest. In fact amylase and its associated polymer substrates have been found to be of considerable interest within the field of polymer therapeutics and specifically the science of conjugating of large, water soluble polymers to bioactive molecules of interest.[74, 75]. Coupling a conventional medicinal to a polymer achieves macromolecular status with distinct therapeutic advantages.[76-78] Polymer therapeutics has yielded some of the major pharmaceutical “block busters” of the past 10 years.[74]

Conjugation to a polymer "shields" the body from potential adverse effects of a payload, as well as the payload itself from being degraded, immunologically attacked, or cleared by the kidneys.[79] Additionally, however, use of a biodegradable polymer with the appropriate enzyme would result in biodegradation and release of the active payload. In this strategy, the biodegradable polymer shields the payload while in transit. Macromolecular status imparts passive targeting of the drug through the EPR effect [31, 77, 80, 81] allowing accumulation at a site of inflammation. The target enzyme present therein will degrade the polymer releasing the active compound. This approach is termed the Polymer Mask Unmask Protein Principle (PUMPT).[82]

The rate of degradation of this polymer conjugated to a drug of choice can be predicted *in vitro* and *ex vivo* by custom-engineering the chain length of the polymer and the degree of pendant groups.[76] Amylase is an endogenously produced enzyme with multiple polymer substrates that happen to be already licensed for human use. PUMPT was in fact described using amylase and succinoylated dextrin as a proof of principle.[81] The PUMPT approach has been described for several clinical applications.

Hardwicke *et al.* used the biodegradable polysaccharide dextrin and a recombinant human epidermal growth factor (rhEGF) as a first in class proof of concept based on the premise that the conjugate would localise to the wound due to the EPR effect.[83] In 2014, a prototype dextrin-colistin conjugate was customised by polymer modification and binding chemistry to afford selective, controlled release at an infected site was reported.[84, 85] These studies employed the biodegradable, naturally-occurring polymer, dextrin, and different bioactive molecules. [82, 84, 86, 87]

While the EPR effect has been demonstrated to be highly successful in pre-clinical species it has been less successful clinically; therefore, additional levels of complexity to augment targeting have been

described. One strategy is to add an antibody binding. Tilmanocept is one such example. Tilmanocept is a recently described, mannosylated, dextran-based polymer therapeutic for sentinel lymph node imaging which may offer an innovative solution for patients with melanoma and breast cancer. It has been FDA approved and has not been associated to any of adverse effects. Tilmanocept binds tightly to CD206 mannose receptors on the surface of reticuloendothelial cells resident in lymph nodes for up to 30 h.[88] In two rigorously conducted phase III trials, Tilmanocept identified correctly a significant proportion (an additional 20%) of melanoma-positive sentinel nodes that were not detected by blue dye.[89] Complexity can also be achieved by loading many biological payloads onto a polyfunctional polymer,[90] such as hydroxyethyl starch conjugation to hemoglobin.[91] Of note, hydroxyethyl starch is a substrate of amylase, and was, until recently, used as a volume expander and carried an increased risk of renal dysfunction and mortality over a 90-day follow-up in patients who received HES compared with crystalloids.[92] Increased mortality in patients with sepsis was also observed prompting their UK-wide recall.[92, 93] It is likely, however, that these adverse effects would not be associated to the quantities administered for drug delivery, which are minute compared to those required for plasma volume expansion.

The efficacy of such targeting techniques does, however, depend on the underlying principles being widespread phenomena, with sufficient magnitude of effect to allow clinical harnessing.[81, 94]. Several studies have focussed on producing novel bioresponsive classes of drugs based on polymer substrates of amylase, but no studies have proven conclusively that amylase actually localises at the site of interest. Application of the same technology to areas where first generation (non-biodegradable) polymer therapeutics have already reached the clinic may have wide-reaching implications for other aspects of surgery, including rheumatoid arthritis, sentinel lymph node identification, and breast cancer.[75, 78]

3.5.2 Other clinical therapeutic uses of amylases in surgery

α-amylase is a component in several pharmaceutical enzyme replacement preparations, used to treat pancreatic insufficiency. For instance, due to the absence of the cystic fibrosis trans-membrane regulator (CTFR), the secretion of α-amylase is obstructed.[95] This means patients with cystic fibrosis can be treated with enzyme replacement therapies to prevent malnutrition.[96] Conversely, α-amylase inhibitors have been licensed for the treatment of type 2 diabetes. Acarbose, one such example, is a pseudotetrasaccharide from Actinoplanes spp. which works by binding to the active subsites -3 through to +2.[97, 98] Similarly, phaseolamin has been marketed as an enteric amylase inhibitor adjunct to weight loss and is available as an over the counter drug. De Gouveia *et al.* found that patients treated with phaseolamin did not have any decrease in body weight, but this study was conducted for only 20 days and therefore, follow-up bias could have affected the conclusions of this study.[99] The use of phaseolamine as an antihyperglycemic is contended. Oliveira *et al.* found that phaseolamin can be used to control diabetes due to its anti-hyperglycemic effect.[100] Another study did not find any effect on the glycemic state, although this could be due to the low dose used in the latter study.[101] Interestingly, the product of amylase activity (glucose) has been shown to inhibit effectively the production of the main toxins responsible for the onset and progression of gas gangrene, lending some credence to claims of antibacterial efficacy of sugar based wound dressings such as Manuka Hone.[102]

3.5.3 Clinical Diagnostics

Hitherto, the principal use of α-amylase remains in clinical diagnostics. The normal range of human serum α-amylase activity is 23-85 IU/L, however there is substantial inter-laboratory and inter-assay variation.[86] Pancreatic α-amylase is produced exclusively by the pancreas which increases its specificity for the diagnosis of pancreatic diseases. Furthermore, detecting the activity of the pancreatic isoform

compared to the total amylase, increases the sensitivity, therefore differentiating between the two isoforms is important clinically.[103, 104] Various methods to accomplish this differentiation have been described previously in literature, including enzyme-immunoassays and partial inhibition of salivary amylase by various different inhibitors.[104] Widespread clinical adoption of classic diagnostic and, more recent therapeutic and combined approaches using α-amylase are dependent on rapid, cost-effective, and real-time quantification of α-amylase activity. A detailed description of state of the art sensors under development or fully developed is provided in the supplementary material (online only). The methods of detection either depend on product formation or substrate degradation or antibody binding. Total α-amylase is used as an indication of pancreatic or salivary disease.

The p-nitrophenol assay is directly proportional to α-amylase activity and is measured photometrically. The range of detection is 3-1500 IU/L. This assay is used widely in the National Health Service (in the United Kingdom), but it does not distinguish bacterial from human amylase.[105] In contrast, antibody assays employ a human anti-α-amylase antibody as it will eliminate any false positives due to bacterial secretion of amylase, while also being sub-type specific.[106] Commercially available assays all rely on measuring activity of α-amylase, while an antibody approach would quantify concentration of α-amylase (as opposed to amylase activity) with a high degree of sensitivity and specificity in control and patient samples. Aluoch *et al.* designed a novel amperometric biosensor using a salivary anti α-amylase antibody which is monitored by an electroactive indicator. This indicator is oxidized or reduced causing current change which gives analytic information on the concentration of amylase. Its limit of detection is 1.57 pg/mL which is more sensitive than enzyme-linked immunosorbent assay (ELISA, 10 ng/mL).[106] Although this study describes a simple design, silver precipitation can be a concern because it would lead to insensitivity, possibly limiting this approach to single use, and thereby markedly increasing cost. An experimental “online” *in vivo* sensor reported by Wu *et al.* uses starch immobilized on a magneto-elastic

sensor. In the presence of amylase, there is a decrease in mass loading resulting in an increase in resonance frequency; the device suffers currently from lack of sensitivity.[107] Nonetheless, this study highlights the potential of rapid, "online" bedside amylase detection methods and offers substantial improvement including cost-effectiveness, and real time measurement. Wang *et al.* developed a protocol which uses a portable device to measure α-amylase concentration. The feasibility of point-of care monitors has been well-established in other clinical conditions such as diabetes.[108] The methodology of Wu et al. however, requires the sample to be incubated for 15 minutes at 37^{o}C for the reaction to occur, which limits the applicability of this method at the point of care, because the sample has to be processed using laboratory equipment before using the sensor. Given the direction of research into amylase as a trigger for several personalisable and therapeutics such as infection, cancer and wound healing, the literature reviewed in this section suggests that miniaturised, cost-effective, personal point-of-care biosensors for α-amylase would have potential advantages over current diagnostic approaches. By being able to record amylase activity at a personal, point of care level, such devices may increase the scope of amylase-based therapeutics, expand both diagnostic and therapeutic use into personalised medicine.

4. Conclusion

Novel amylase-based therapeutics and diagnostics have experienced a period of intensive research, resulting in novel technologies with substantial clinical potential. Clinically relevant classifications of hyperamylasemia presented in this study may help to direct the surgeon during the "surgical sieve" diagnostic process. Proof-of-concept for the EPR and the PUMPT phenomena may provide novel and bioresponsive "smart" approaches to the management of common surgical disease. These advances are based on interface research linking demand (clinical) to supply (basic sciences), providing novel and exciting solutions to surgical problems of global health concern, and it is, therefore, important for the practicing surgeon to be aware of progress in this field. The advent of portable point-of-care, amylase

sensing devices may facilitate surgical decision making and applicability of amylase-based polymer therapeutics. Further availability of *ex vivo* and clinical data to support the use of amylase-based therapeutics would support allow clinical harnessing, and stimulate further development of novel, effective drugs based on such principles.

Abbreviations:

PUMPT Polymer Mask Unmask Protein Therapy Principle. CAZy = carbohydrate-active enzymes; EPR: Enhanced permeability and retention; PUMPT = polymer mask-unmask protein therapy; CFTR = Cystic Fibrosis Transmembrane Regulator, HIV = Human Immunodeficiency Virus, MetS = Metabolic syndrome; AMP = Amperometric, EIS = Electrochemical Impedance Spectroscopy, FAU = Fungal Amylase Unit, FIA = Flow Injection Analysis, KNU = kilo Novo unit, LOD = Limit of Detection, LR = Linear Range, nkat = nanokatals.

Competing interests:

The authors declare no conflict of interest.

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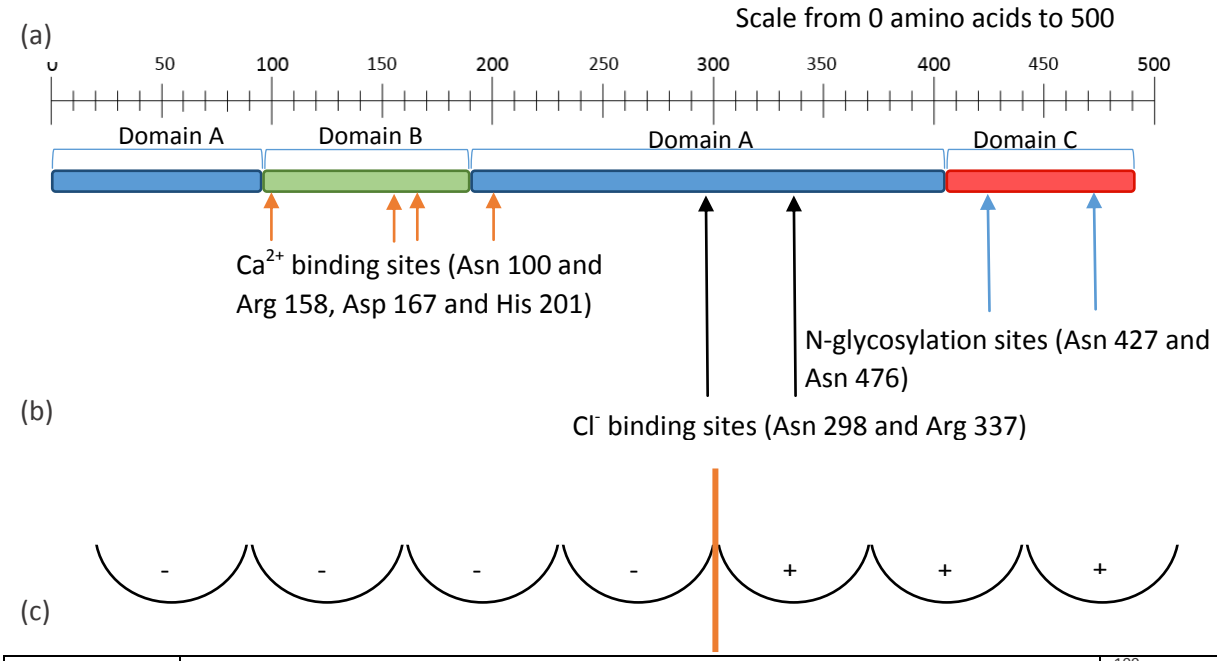
Figure Legend

Figure 1: Systematic representation of the structure of human α-amylase (a) Amino acid sequence of human α-amylase indicating the binding sites for calcium (orange arrows), chloride (black arrows) and N-glycosylation sites (blue arrows) (b) Active site is a V-shaped cleft subdivided into different sub-sites, glycone, individual sugar residues in a oligosaccharide chain (-4,-3,-2 and -1) and aglycone, individual sugar residues after hydrolysis of glycosidic bond (+1, +2 and +3). Each sub-site interacts with a monosaccharide, and cleavage occurs between +1 and -1, shown in the figure by the orange line.

Figure 2: Systematic representation for the hydrolysis of glycosidic linkages by amylase. (a) The first displacement mechanism. The acid group of α-amylase reacts with the glyosidic oxygen to form an oxcarbenium ion-like transition state. This goes through nucleophilic attach by the acid of α-amylase to give a β-glycosyl enzyme intermediate. (b) The β-glycosyl enzyme intermediate reacts with activated H_2O which forms an oxcarbenium ion-like transition state. The acid group of α-amylase is then reprotonated. Most forms of α-amylase are endo-enzymes, acting on substrate bonds within the carbohydrate chains, rather than those at the terminal ends. In contrast, maltogenic amylase is an exoenzyme subclass which hydrolyses terminal end residues which lead to them being re-classified as maltogenic amylases.



Number of Genes	Two pancreatic α-amylase genes and one salivary α-amylase gene, at chromosome 1p21.	[109]
Evolution	Salivary amylase gene has evolved from a pre-existing pancreatic amylase gene. The alteration in tissue specificity is probably due to retroviral insertion upstream of the amylase gene	[109]
Domain structure	Domain A (residues 1-99, 170-404) has an eight-stranded parallel β-barrels in the centre of α-helical segments. This αβ architecture forms different subsites of the active site (Figure 1b.). Domain B (100-168) forms a large loop between the third β-strand and the α-helix. Domain C (405-496) consists of a β-stranded structure (Figure 1a.)	[19, 110, 111]
Homology	Salivary and pancreatic α-amylase have 97% homology with each other. A total of 15 individual amino acid substitutions result in three more polar groups and three side chains to the salivary amylase	[19, 110]
Functional significance	Six of these individual substitutions are between residues 340-404 which is within the amino acid sequence that connects the A domain and C domain.	[19]
Clinical application	Two of these substitutions Thr 163 →Ser and Leu 196 →Ile have a direct effect on substrate binding, and different specificities to length of polysaccharides.	[19]
Post translational modifications	In pancreatic α-amylase, a carbon atom on the side chain of the terminal glutamine residue and increases stability by providing protection against amino peptidases. This post-translational modification appears to be specific to pancreatic (as opposed to salivary) amylase.	[19]

Figure 1: Systematic representation of the structure of human α-amylase (a) Amino acid sequence of human α-amylase indicating the binding sites for Calcium (orange arrows), Chloride (black arrows) and N-glycosylation sites (blue arrows) (b) Active site is a V-shaped cleft subdivided into different sub-sites, glycone, individual sugar residues in a oligosaccharide chain (-4,-3,-2 and -1) and aglycone, individual sugar residues after hydrolysis of glycosidic bond (+1, +2 and +3). Each sub-site interacts with a monosaccharide and cleavage occurs between +1 and -1, shown in the figure by the orange line. (C) Important difference from human pancreatic to salivary amylase are listed herein.

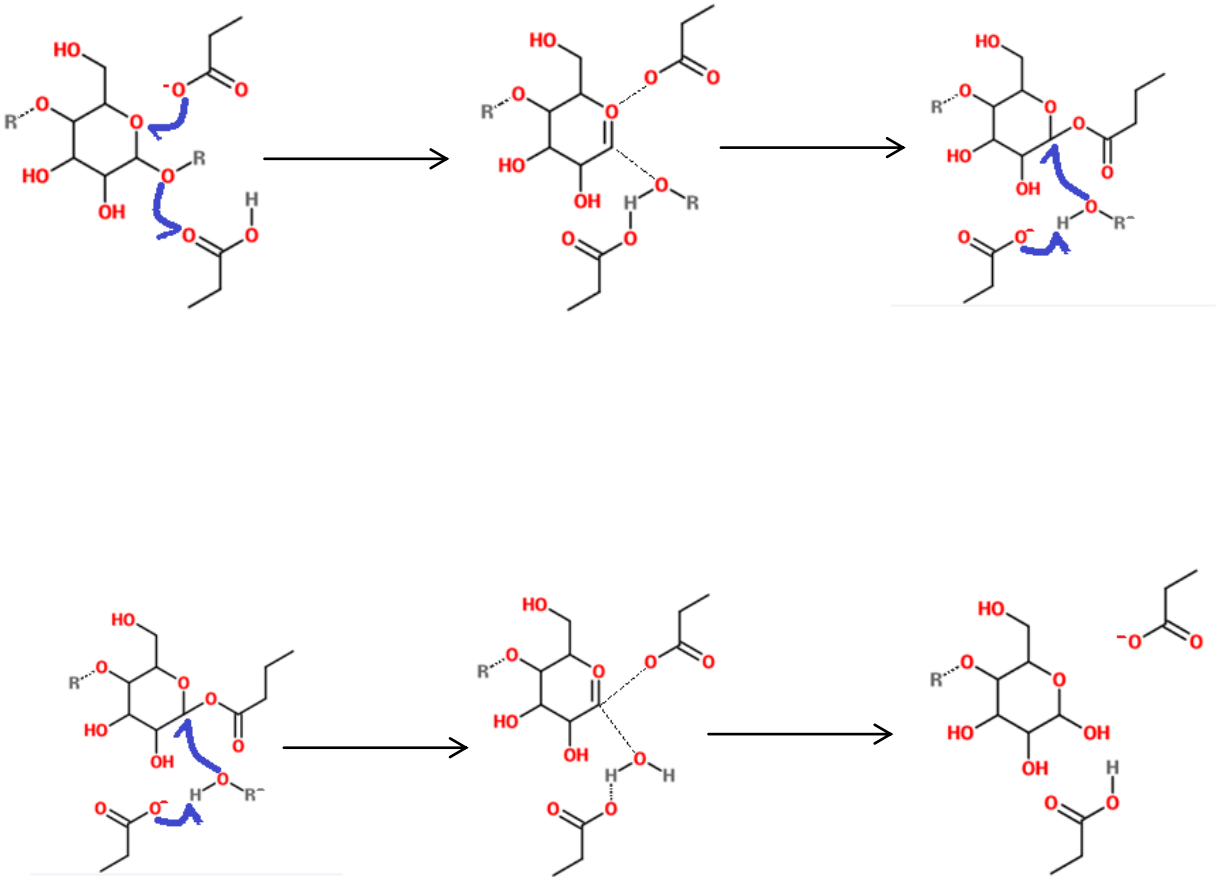


Figure 2: Schematic representation for the hydrolysis of glycosidic linkages by amylase. (a) The first displacement mechanism. The acid group of α-amylase reacts with the glyosidic oxygen to form an oxcarbenium ion-like transition state. This goes through nucleophilic attach by the acid of α-amylase to give a β-glycosyl enzyme intermediate. (b) The β-glycosyl enzyme intermediate reacts with activated H_2O which forms an oxcarbenium ion-like transition state. The acid group of α-amylase is then reprotonated. Most forms of α-amylase are endo-enzymes, acting on substrate bonds within the

carbohydrate chains, rather than those at the terminal ends. In contrast, maltogenic amylase is an exoenzyme subclass which hydrolyses terminal end residues which lead to them being re-classified as maltogenic amylases.

Tables

Table 1: Current classifications of Amylolytic Enzymes

Subtheme	**Current Trends in Literature**
Homology	Minimal amino acid sequence homology but high homology in polypeptide folding. Example: α-amylase very similar active site centred around three conserved carboxylate groups. Four segments with of sequence with good homology; 96-101, 193-201, 233-236 and 294-301.[112, 113]
Classification method	IUB method does not take into account evolutionary events. Amylolytic enzymes characterized on amino acid sequence homology, reaction mechanism, three dimensional structure and active sites.[15, 16]
Number of families	1991 found 35 different glycoside hydrolase families looking at sequence homology [15]. Over 950 glycosyl hydrolases have been sequenced [113-115] to date.
Family similarities	Three dimensional structure suggests that families may be related to each other. For example, families 19, 22, 23 and 24.[115]
CAZy database	Contains family divisions based on amino acid sequence of all CAZymes. In 2009, the database covered 300 protein families including glycosyltransferases, polysaccharide lyases and glycoside hydrolase.[116] In 2013, there were 330 families and over 340000 CAZymes with sequence information.[117] α-amylase falls into the glycoside hydrolase family 13 [110].

CAZy = carbohydrate-active enZymes

Table 2: Pathophysiological states affecting plasma amylase levels other than pancreatic and salivary

Disease	α-amylase activity	Study
Diabetes Mellitus	A study of 9340 diabetics 1102 (11.8%) had elevated α-amylase of that, 22.3 (4.1 %) had abnormal renal function.	[118]
Sjögren's Syndrome	24% of patients with Sjögren's syndrome have persistent hyperamylasemia of either isotype, pancreatic or salivary. Related to a moderate subclinical inflammatory progression.	[119] [120]
Inflammatory Bowel Disease	Increased serum amylase after treatment of ulcerative colitis by azathioprine. Serum amylase was increased (149 U/L) but amylase was absent in the urine. This could potentially indicate the presence of macroamylasemia. Another case report showed similar results.	[121, 122]
Renal Failure	This may be due to a decreased clearance level of the kidneys. Hussein *et al.* thought that elevated serum amylase could be due to pancreatic damage due to reactive oxygen species. Renal failure increases the oxidative stress and inflammatory states of the pancreas.	[123, 124]
Peptic Ulcer	Motamedi *et al.* found salivary amylase activity to be increased, possibly because of oxidative stress from the peptic ulcers.	[125-127]
Non-alcoholic Fatty Liver Disease	Manifestation of a metabolic syndrome and insulin resistance. It has been shown to be related to low serum amylase levels independent of MetS, type 2 diabetes, and obesity.	[23]
Coeliac	Coeliac disease linked to hyperamylasemia and macroamylasemia	[24, 25]
Familial Hyperamylasaemia	Gullo *et al.* first described this condition from studying 18 patients who had increased amylase levels for a mean of 7.6 years. Two case studies were next to describe this condition in two young boys with abdominal pains and increased serum amylase. Both concluded it to be familial hyperamylasaemia and it to be autosomal dominant.	[128-130]
Chronic Physiological Stress	Increased serum amylase have been reported with increased stress, however, results are contended.	[131-134]
Acute HIV	A study of 94 HIV patients 7.5 (8%) patients have increased serum α-amylase pre-treatment, versus a study of 1368 HIV patients, 916.5 (67%) patients had increased serum α-amylase activity peri/post treatment, suggesting this increase is partially due to treatment options. Amylase activity may be as increased as 535 IU/L.	[135-138]
Bacterial Infection	Bacillus, Clostridium and Aeromonas spp. constitutively produce amylase.	[139, 140]

**Variations in amylase activity with pancreatic disease are extensively discussed elsewhere.*
CFTR = Cystic Fibrosis Transmembrane Regulator. MetS = Metabolic syndrome

Supplementary material: State of the art in currently available amylase detection technique

Assay	Design	Detection		Limits of detection	Specificity	Ref.
		Sequential Process	Technique			
Rate of product formation	g a ıle and ıe with α-glucosidase and optionally mutarotase cross-linked by gelatin-glutaraldehyde	α-glucosidase converts maltose to α-D-glucose. Mutarotase converts α-D-glucose to β-D-glucose which is determined via glucose oxidase	AMP	LOD: 2nkat/mL (0.117.64 units/mL) when reaction time 5 min 0.5 nkat/mL (0.02941 units/mL) when reaction time 30 min LR: 0.1-3 mmol/L	Any α-amylase	[141]
	Screen-printed electrodes with immobilized α-glucosidase, glucose oxidase and mutarotase modified with Prussian Blue	α-glucosidase converts maltose to α-D-glucose. Mutarotase converts α-D-glucose to β-D-glucose which is determined via glucose oxidase	AMP	LOD: 5 units/mL LR: 5-250 units/mL	Any α-amylase	[142]
	Flow-injection device using maltopentaose as substrate. α-glucosidase immobilised on pre-activated membrane. Glucose oxidase immobilized on electrode	α-glucosidase converts maltose to 5-d-glucose. Glucose oxidase coverts 5-d-glucose to gluconic acid and hydrogen peroxide which is measured	AMP	LOD: LR: 0-30 units/mL	Any α-amylase	[143]
	Spectrophotometric flow injection measuring brick red complex formation at 540nm	Amylose incubated with sample to produce maltose. 3,5 dinitrosalicylic-acid and maltose boiled	SIA/FIA	LOD: 0.0048 FAU LR: 0.005-0.06 FAU	Any α-amylase	[144]
	Use of portable personal glucose meter	Sample, α-glucosidase and maltopentaose incubated 15 min at 37^0C.		LOD: 20 U/L LR: 2.2-27.8 mM	Any α-amylase	[145]
	Flat-chip micro analytical sensor used as part of a Micro-Electro-Mechanical Systems. Pre-column and flat-enzyme electrode incorporated into a flow cell where maltose	Maltose phosphorylase phosphorylates maltose. Glucose oxidase converts phosphorylated maltose to gluconic acid and	Electrochemical and Lateral flow	LR: 0-190 kU/L	Any α-amylase	[143]

	phosphorylase, glucose oxidase and peroxidase immobilised	hydrogen peroxide which is measured				
	Colorimetric assay biosensor system using Gal-G2-CNP, chromogenic substrate for α-amylase. CNP is a yellow product once hydrolysed which can be measured photometrically at 430nm.	Disposable test strip placed under tongue (25 µl). Once strip inserted into reader and saliva moved onto the reagent paper. The entire test takes roughly 30 sec.	AMP	LR: 10-230 U/mL	Any α-amylase	[146]
Rate of starch digestion	:ric starch-iodine complexes	Sample degradation of complexes measured in flow channel	FIA	LOD: 60 NU/mL LR: 0.25-5.0 KNU/mL	Any α-amylase	[147]
	Immobilized layer of starch gel on thick-film magneto elastic sensor and presence of α-amylase alters the resonance frequency	Sample placed on starch gel		LR: 75-125 U/mL	Any α-amylase	[148]
	Spectrofluorimetric using the quenching of luminescence intensity (634nm) of nano CdS doped in sol-gel of different concentrations of maltose	Sample incubated with starch in flow channels	FIA	LOD: 5.7 x 10^{-11} mol/L LR: 4.8 x 10^{-10} – 1.2 x 10^{-5} mol/L	Any α-amylase	[149]
	Glycogen/amylopectin spin-coated on gold coated quartz crystals (case frequency of 10 MHz). Films cross-linked with hexamethylene diisocyanate. Film degradation measured with quartz crystal microbalance	Sample incubated with film	EIS		Any α-amylase	[150]
	Degradation of starch-triiodide measured using platinum redox sensor for direct potentiometric determination	Sample incubated with starch-triiodide		LOD: 1.944 mU LR: 0-0.54 U	Any α-amylase	[151]
	Glucose oxidase-based biosensor measuring the decrease in dissolved oxygen concentration related to starch concentration.			LOD: LR: 0.66-9.83 U/mL	Any α-amylase	[152]

	Glutaraldehyde as a cross-linker				
Antibody to antigen interactions monitored by an electroactive indicator ($K_3Fe(CN)_6$).	The electroactive was oxidized or reduced depending on concentration of salivary α-amylase present	AMP	LOD: 1.57 pg/mL LR: 0.003-0.016 ng/mL	Human salivary α-amylase	[106]

AMP = Amperometric, EIS = Electrochemical Impedance Spectroscopy, FAU = Fungal Amylase Unit, FIA = Flow Injection Analysis, KNU = kilo Novo unit, LOD = Limit of Detection, LR = Linear Range, nkat = nanokatals

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