

## Purification, properties, and characterization of amylase: a review

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**Abstract.** Amylase is a biological macromolecule that catalyzes the hydrolysis of starch or glycogen. It has a wide range of applications in various fields such as sugar making, pulp making, textiles, and medicine. There are different types of amylases, with  $\alpha$ -amylase and  $\beta$ -amylase being the most common. Additionally, the structures and catalytic properties of amylases produced by different species vary. This article reviews the purification, enzymatic properties, and structural characterization of amylase, providing a reference for clarifying the catalytic characteristics of amylase and better controlling its catalytic reaction.

**Keywords:** amylase; column chromatography; electrophoretically pure; optimum reaction conditions; molecular modeling; structure prediction.

### 1. Introduction

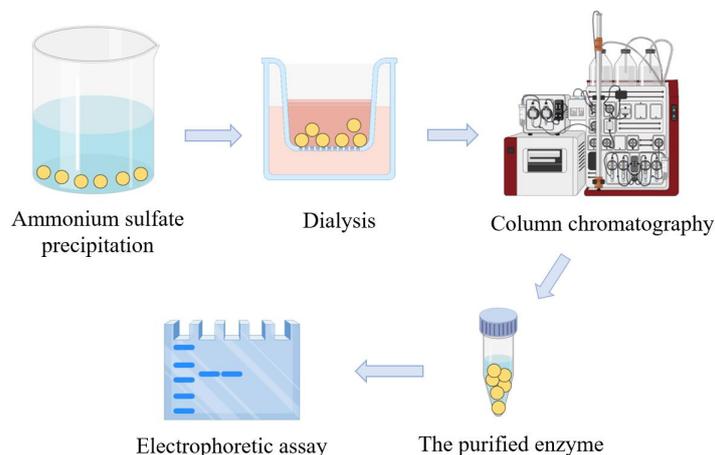
Starch and glycogen serve as energy storage substances for organisms, and their final hydrolysis product, glucose, can produce ATP through glycolysis and the tricarboxylic acid cycle pathway to provide energy. Amylase refers to a group of enzymes that can break down starch or glycogen. Interestingly, amylase was the earliest biocatalytic macromolecule discovered by humans. In 1833, *Payen* and *Persoz* first obtained biocatalytic macromolecules with the function of decomposing starch<sup>[1]</sup>; In 1924, *Kuhn* divided these biocatalytic macromolecules into two categories, namely,  $\alpha$ -amylase and  $\beta$ -amylase, which are respectively it works by releasing  $\alpha$ - and  $\beta$ -type maltose<sup>[2]</sup>; In subsequent studies, scholars also discovered  $\gamma$ -amylase<sup>[3]</sup> and isoamylase<sup>[4]</sup>.

Amylase can decompose starch efficiently and safely, thus it is widely used in the field of sugar processing, which is mostly found in the brewing, baking, and fruit and vegetable processing industries<sup>[5]</sup>. In addition to these applications, amylase has garnered significant interest from scholars worldwide due to its various uses in pulping, textiles, chemicals, medicine, and other fields<sup>[6]</sup>. The molecular structures and catalytic properties of amylases from various sources can differ significantly<sup>[7]</sup>. To gain a clearer understanding of the enzymatic properties of the specific amylase being studied and to better control its catalytic reactions, it is essential to isolate and purify it. Furthermore, shedding light on its molecular structures is crucial to explore its catalytic mechanisms.

### 2. Purification of amylase

The essence of amylase is protein, and its purification method is similar to that of protein purification. but it requires the detection of amylase enzyme activity at various isolation purification steps to ensure the effectiveness of the purification operation. Commonly used amylase purification

methods are protein precipitation (including salting out and alcohol precipitation, etc.), membrane filtration, column chromatography (including ion exchange chromatography and gel filtration chromatography, etc.), gel electrophoresis, etc. The methods of protein precipitation and membrane filtration serve to concentrate amylase initially, but for further purification, column chromatography is necessary. Once this step is completed, the effect of the purification process and the presumed molecular weight can be assessed through gel electrophoresis (Fig. 1). *Jia et al.*<sup>[8]</sup> used membrane filtration technology to extract  $\beta$ -amylase from sweet potato starch waste solution, and concentrated  $\beta$ -amylase 5.6 times under the conditions of pH 7.0, 20 °C, operating pressure 0.14 MPa, and molecular weight cut-off of 50 kDa; On the basis of ammonium sulfate precipitation and dialysis, *Zhang et al.*<sup>[9]</sup> first concentrated the sea cucumber body wall extract by DEAE-52 anion exchange chromatography column and then separated the active concentrated solution by Sephacryl S-300 gel chromatography column to obtain electrophoretic purity  $\alpha$ -amylase with a molecular weight of approximately 420 kDa; *Van et al.*<sup>[10]</sup> and *Jia et al.*<sup>[11]</sup> also combined the DEAE anion exchange chromatography column and the Sephacryl gel chromatography column to obtain electrophoretically pure amylase from fermentation products of *Bacillus.sp* FW2 and *Aspergillus oryzae* FAFU, respectively, with molecular weights of 55 kDa for the former and 48.6, 49.7, 93.5 kDa for the latter; *Yan et al.*<sup>[12]</sup> performed DEAE-52 anion exchange chromatography column pre-separation on the crushed solution of *Bacillus pumilus* HB-3 before dialyzing the active separated solution with polyethylene glycol-20000, and then the concentrated solution was further purified by CM-52 cation exchange chromatography column to obtain an electrophoretically pure amylase of 56.5 kDa. According to the above literature, a combination of column chromatography techniques employing two separation principles may be needed to achieve the electrophoretic purity of amylase. In addition, the amylases from different sources vary in molecular weight size, and the types of amylases from a single source may not be unique.



**Figure 1** Purification steps of amylase

(Figure material was provided by figdraw, <https://www.home-for-researchers.com/static/index.html/#/>)

### 3. Enzymatic properties of amylases

An enzyme is essentially a protein with a catalytic function, whose activity can be influenced by various factors including temperature, pH, activators, and inhibitors<sup>[13]</sup>. Exploring the enzymatic properties of amylases is an effective way to control their catalytic efficiency and expand their range of applications.

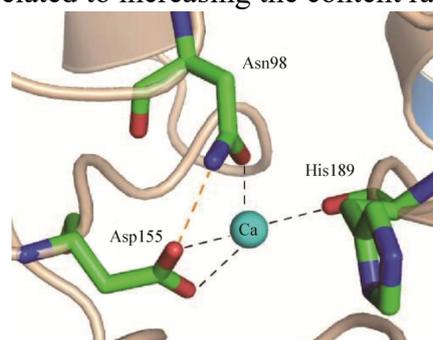
When it comes to the catalytic system of amylase, temperature and pH are crucial environmental factors that need to be taken into account, as they are closely linked to the practical applications of amylases. The optimum reaction temperature and pH of amylases tend to vary greatly depending on their source of origin. Table 1 provides examples of the optimum reaction temperature and pH for various sources of amylase.

**Table 1** Optimum reaction temperature and pH of amylase from different sources

Source	Enzyme	Optimum reaction conditions		Reference
		Temperature/°C	pH	
Bacillus sp. Amy-43	amylase	75	4.0	[14]
Bacillus licheniformis RA31	$\alpha$ - amylase	70	8.0	[15]
Paederia foetida	$\beta$ - amylase	60	6.0	[16]
Bacillus pacificus	$\alpha$ - amylase	55	9.0	[17]
Portunus segnis	$\alpha$ - amylase	45~55	7.5	[18]
Cladophora hutchinsiae	amylase	10	6.0	[19]

The study of enzyme activators and inhibitors revolves around identifying the ideal reaction and stable conditions for enzyme activity. It's worth noting that the effects of activators and inhibitors are not fixed. For instance, low concentrations of NaCl can enhance amylase activity, while high concentrations can result in protein dehydration, leading to reduced viability.

Certain inorganic salt ions are known to be effective activators of amylases. According to molecular modeling (Fig. 2),  $\alpha$ -Amylase is a catalytic enzyme that relies on  $\text{Ca}^{2+}$  for its activity. This is due to the fact that  $\text{Ca}^{2+}$  can offset the electrostatic repulsion between the Asn120, Asp200, and His233 carbonyl oxygen within the  $\alpha$ -amylase domain, thereby increasing its stability<sup>[20]</sup>. In addition to  $\text{Ca}^{2+}$ , less than 60 mg/kg of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Cu}^{2+}$  is considered to enhance the enzyme activity of  $\alpha$ -amylase and  $\beta$ -amylase of barley<sup>[21]</sup>. Among inorganic non-metallic ions, the appropriate concentration of  $\text{Cl}^-$  has been proven to have an activation effect on amylase, and the mechanism of its effect may be related to increasing the content ratio of  $\beta$ -turn and random coil<sup>[22]</sup>.



**Figure 2** Protein structure simulation of the calcium ion-binding domain of  $\alpha$ -amylase from *Tenebrio molitor* larvae<sup>[20]</sup>.

Compared to activators, amylase inhibitors have a greater number of literature reports. Effective amylase inhibitors have been found to reduce blood sugar levels, prevent insect pests, and so on<sup>[23,24]</sup>. These inhibitors can be classified into protein and non-protein inhibitors. Protein inhibitors can be classified into two types based on their mechanism of inhibition<sup>[25]</sup>. The first type involves the inhibitor binding to the catalytic group of amylase through hydrogen bonds or forming a hydrogen bond network of amylase-water-inhibitor by associating with surrounding water molecules. The second type involves connecting the hydration  $\text{Ca}^{2+}$  on the surface of the inhibitor to the catalytic site of amylase. Non-protein inhibitors encompass a wide range of compounds such as

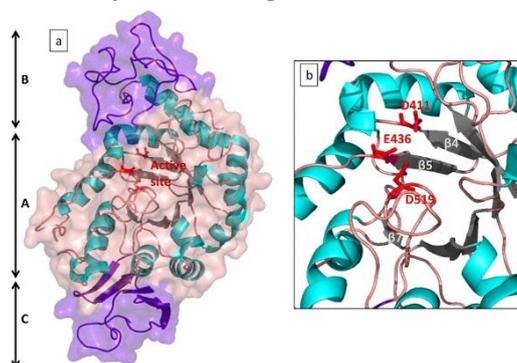
metal ion chelating agents, heavy metal ions, polyphenols, flavonoids, and phenolic acids. The inhibitory effects of metal ion chelating agents and heavy metal ions (such as  $\text{Ce}^{3+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$ , etc.) on amylase are related to robbing and replacing  $\text{Ca}^{2+}$  in the amylase domain to change its conformation, respectively<sup>[26]</sup>. The inhibition mechanisms of polyphenols, flavonoids, and phenolic acids on amylase are similar. Specifically, the phenolic hydroxyl groups of polyphenols, the hydroxyl groups on flavone benzopyrans, and the hydroxyl and methoxyl groups on the aromatic rings of phenolic acids can all form hydrogen bonds with the amino acid side chains found in the active sites of amylase<sup>[27]</sup>. These substances' aromatic rings can occupy the active sites via hydrophobic interactions, effectively shielding them from contact with the substrate<sup>[28]</sup>.

#### 4. Structural characterization of amylases

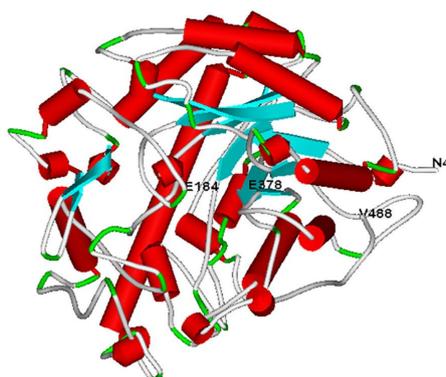
For further clarity on the catalytic characteristics and enzymatic mechanism of amylases, structural characterization of them is required. X-ray crystal diffraction and nuclear magnetic resonance are two methods commonly used for protein structure characterization. These techniques have been utilized to analyze the structure of tens of thousands of proteins. However, they are not frequently utilized today due to certain limitations. X-ray crystal diffraction requires proteins to be crystallized, which can be a tedious and time-consuming process. Additionally, some proteins cannot be crystallized. On the other hand, nuclear magnetic resonance has a molecular weight limit (typically below 30 kDa) for the objects it can analyze. To rapidly characterize the structure of proteins, structural prediction technology was developed and quickly advanced.

There are three current methods for protein structure prediction: homology modeling, threading, and ab initio<sup>[29]</sup>. Homology modeling is based on the assumption that proteins with similar sequences also have similar spatial structures. To determine if a protein can be homology modeled, the similarity between the target protein sequence and the template protein sequence is calculated. If the modeling requirements are met, the spatial structure of the target protein can be constructed in a pairwise manner. The threading method involves aligning the sequence of the target protein with various alternative template protein structures. This process allows for the screening of the best template protein at a structural level. From there, the structure of the target protein can be predicted through structural optimization. Ab initio predictions involve generating several protein structural fragments from the amino acid sequence of the target protein. These fragments undergo splicing and conformational optimization to create a protein prediction model. Of the three methods for protein structure prediction, homology modeling is the most widely utilized. This method can achieve over 90% accuracy in an amino acid pairing when the sequence similarity between the target protein and the template protein is greater than 50%. Furthermore, protein structure prediction is less challenging with this approach<sup>[30]</sup>. Since protein crystallization and amino acid sequencing were not performed during protein structure prediction, the amino acid sequence of the protein can only be deduced from the corresponding gene sequence of the model strain (same species) in the database, and then search for homologous protein sequences or similar protein sequences through protein database matching. Structural proteins are finally modeled and optimized, and the predicted results are only theoretical speculations. Non-Redundant database (<http://ncbi.nlm.gov/protein/>), Protein Database Bank database (<https://www.rcsb.org/>) and SWISS-MODEL server (<http://swissmodel.expasy.org/>) are the most commonly used protein homology sequence library, structure library and homology modeling tools, respectively.

The Carbohydrate-Active Enzyme (CAZy) database (<http://www.cazy.org>) classifies proteins with similar amino acid sequences<sup>[31]</sup>.  $\alpha$ -Amylases belong to the GH119, GH57, and GH13 families of glycoside hydrolases (GH), and  $\beta$ -amylases are assigned to the GH14 family. The pocket-like structure of  $\beta$ -amylase is highly different from the slit-like structure of  $\alpha$ -amylase, indicating that the structural domains of the catalytic active centers of the two are different, which may be one of the reasons for their different catalytic mechanisms. *Hlima et al.*<sup>[32]</sup> performed structural simulations on the  $\alpha$ -amylase (aAmy3470) of *C. vulgaris*, and found three domains (A, B, and C) that are typical of  $\alpha$ -amylases (Fig. 3a). The active center of the enzyme is situated within domain A, with its three catalytic site residues (nucleophile Asp411, acid-base catalyst Glu436, and transition-state stabilizer Asp519) located in the loop following  $\beta$ -strands  $\beta$ 4,  $\beta$ 5, and  $\beta$ 7 (Fig. 3b). *Ben*<sup>[33]</sup> believes that  $\beta$ -amylase has three typical domains, one is the N-terminal domain with  $(\beta/\alpha)$ 8 barrel-shaped catalytic structure, the second domain is a small segment formed by three long loops, and the last one is the C-terminal domain mainly composed of  $\beta$ -sheets. The scholar conducted a simulation of oat  $\beta$ -amylase's three-dimensional structure and discovered that it possesses a core structure resembling a  $(\beta/\alpha)$ 8-barrel (Fig. 4). The enzyme's active site is situated in a bag-shaped cavity at one end of this barrel structure. This arrangement renders the enzyme more readily available to the non-reducing end of the starch, thereby confirming its classification as an exohydrolase.



**Figure 3** The structure (a) of  $\alpha$ -amylase (aAmy3470) and its active catalytic center (b)<sup>[32]</sup>.



**Figure 4** Predictive model of oat  $\beta$ -amylase (AsBAMY) in the GH14 family<sup>[33]</sup>.

In certain practical applications, it may be necessary to investigate the interaction between enzymes and non-substrate substances in solution systems. To achieve this, structural characterization techniques such as ultraviolet-visible spectroscopy, infrared spectroscopy, fluorescence spectroscopy, circular dichroism spectroscopy, and laser Raman spectroscopy can be employed. Among these techniques, fluorescence spectroscopy and circular dichroism spectroscopy are most commonly used, both of which can provide evidence for protein conformational changes. After performing molecular docking of chitoooligosaccharide-epigallocatechin gallate (COS-EGCG)

and  $\alpha$ -amylase, *Mittal et al.*<sup>[34]</sup> used fluorescence spectroscopy to analyze the fluorescence quenching effect of COS-EGCG on  $\alpha$ -amylase, indicating that COS-EGCG would cause the conformational change of  $\alpha$ -amylase thereby inhibiting enzyme activity. *Zhang et al.*<sup>[35]</sup> observed that tea polyphenols have the ability to alter the secondary structure of  $\alpha$ -amylase. Through circular dichroism spectroscopy, they found that the layered structure of the enzyme transforms into a helical shape, resulting in a more relaxed structure thereby inhibiting its catalytic activity.

## 5. Look Into the Distance

The process for purifying amylase is well-established. Once the crude enzyme solution is extracted, it undergoes several steps, including preliminary protein concentration (using techniques like salting out or ultrafiltration), purification (typically through column chromatography), and re-concentration (often accomplished through freeze-drying). These steps ultimately yield a high-purity enzyme powder. High-purity enzymes are primarily utilized in scientific research, medical, and other specialized fields. On the other hand, enzymes intended for industrial use do not necessarily need to be of high-purity, as this would be too costly and unnecessary given the current market demand. In most cases, the standard is only based on enzyme activity. Therefore, the development of appropriate amylase purification methods for each industry can be a promising area for future research.

In addition, not all species' amylases can be expressed with high efficiency under ideal conditions, but amylase production can be achieved by heterologous expression techniques. For example: *Soto-Robles et al.*<sup>[36]</sup> in order to explore the structure and properties of  $\alpha$ -amylase in the intestinal tract of Bark Beetle (Curculionidae: Scolytinae), they expressed it heterologously based on an *E. coli* vector; *El-Sayed et al.*<sup>[37]</sup> also used an *E. coli* vector to express  $\alpha$ -amylase of *Laceyella sp.* DS3 by Heterologous expression in order to efficiently purify amylase and explore its enzymatic properties after immobilization. Many recombinantly expressed proteins can be conveniently purified using nickel columns due to embedding six histidine tags.

The advancement of computer technology has transformed protein structure analysis from conventional methods of purification, crystallization, and detection to contemporary techniques that rely on structural prediction derived from gene or amino acid sequences. Contemporary molecular modelling techniques can offer theoretical explanations for the catalytic or inhibitory mechanisms of amylase. However, these explanations are only considered "reasonable speculations". The development of enzymatic research would be greatly enhanced if there were convenient validation methods available.

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