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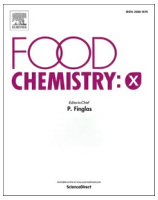

Maleic anhydride-modified xylanase and its application to the clarification of fruits juices

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ABSTRACT

At presently, the catalytic activity of xylanase is sub-optimal, and the required reaction conditions are harsh. To improve its catalytic activity and stability, xylanase (XY) was chemically modified with maleic anhydride (MA). The enzymatic properties of this maleic anhydride-modified xylanase (MA-XY) were then evaluated and analyzed spectroscopically. The results showed that the thermal stability, use of organic solvents, storage stability and the pH range of 3.0 to 9.0 for MA-XY were better than that for XY alone. The kinetic parameters of the enzyme (K_m values) decreased from 40.63 to 30.23 mg/mL. Spectroscopic analysis showed that XY had been modified by the acylation reaction to become a tertiary structure. An assay based on clarifying fruit juices showed that the clarification capacity and reducing sugar content using MA-XY increased compared with those using XY. Overall, this study provides a theoretical basis for improving the application of XY in the food industry.

1. Introduction

Fruit juice, a common food in our daily diet, not only has a pleasant taste but also has a stimulating effect on the digestive system (Kharazmi, Taheri-Kafarani, & Soozanipour, 2020). Juice production is a significant sector of the food manufacturing industry but the formation of by-products and loss of nutrients in juice directly affects its quality and thus its nutritional health benefits to humans (Aadil, Madni, Madni, Raman, & Zeng, 2019). Turbidity in fruit juices caused by colloids containing large amounts of polysaccharide components such as hemicellulose, cellulose and pectin, lead to a loss of quality during its storage and processing (Lucas, Plinho, Roberto, Manuela & Rafael., 2016). Preventing undesirable characteristics, such as turbidity and improving product quality and storage stability, are therefore of great importance in the fruit juice industry. Physical treatments to overcome these problems have certain limitations compared with enzymatic treatment, such as low productivity and altered nutrient content.

Enzymes act as biocatalysts that reduce the activation energy of reactions and make them proceed more rapidly. Xylanase (EC 3.3.1.8) is a hemicellulolytic enzyme from the hydrolase family that hydrolyzes xylan. It can significantly improve the shelf life and safety of fruit juices (Alagöz, Varan, Toprak, Yildirim, Tukel, & Fernández-Lafuente, 2021). Xylanase can liquefy fruit, reduce viscosity and thus increase juice aroma and stability, thereby increasing the juice yield (Rosmine, Sainjan, Silvester, Alikkunju, & Varghese, 2017). XY has been reported to have a beneficial effect on the human microbiota by reducing the viscosity of intestinal chyme (González-Ortiz, Lee, Vienola, Raatikainen, Jurgens, Apajalahti, & Bedford, 2022). It can also improve the nutritional value of animal feed products and the immune activity of plants (Anand, Leibman-Markus, Elkabetz, & Bar, 2021), thereby offering various potential industrial applications. XY can also contribute to the quality of paper bleaching by increasing brightness and reducing lignin content (Sharma, Chaudhary, Kaur, & Arya, 2020). However, there are many practical problems regarding the industrial use of enzymes, which can reduce their activity and stability (Mostafa, El Aty, Hassan, & Awad, 2019). Water is often used as a base solvent in reactions catalyzed by enzymes (Joao et al., 2019). However, because most protein groups contain non-polar amino acids, the non-polar atomic clusters on the surface of the protein spheres become extremely unstable after contact with water, thus rendering water a poor solvent under certain practical conditions (Ishizuka, Chapman, Kuchel, Coureault, Zetterlund, & Stenzel, 2018). Therefore, organic solvents can enhance enzymatic activity (Stepankova, Bidmanova, Koudelakova, Prokop, Chaloupkova, & Damborsky, 2013). However, the used of organic solvents instead of water

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can reduce or promotes catalytic activity, thus decreasing catalytic efficiency (Noriyuki, Doukyu, Hiroyasu, & Ogino, 2010). The catalytic efficiency of XY is sensitive to reaction conditions, causing instability which requires costly storage conditions. Research aiming to improve the enzymatic properties and stability of XY has thus become a topic of increasing interest.

Enzymes can be modified using three main methods: non-buffered salt can be added to the enzyme, after which the enzyme can be lyophilized; the enzyme can be modified via genetic engineering; and enzyme molecules can be chemically modified. Of these methods, chemical modification has been most widely studied and is considered adequate, because it can improve the catalytic activity, stability, and protein resistance of enzymes (Liang, Zhang, Ran, Su, Jiao, Feng, & Liu, 2018). Traditional methods of chemical modifications rely on nucleophilic amino acids, mainly cysteine or lysine residues (Adusumalli, Rawale, Hakur, Purushottam, Reddy, Kalra, & Rai, 2020).

As an enzyme modifier, acid anhydride can enhance enzyme stability by modifying the lysine residues on the enzyme surface, resulting in charges in change (Danait-Nabar, & Singhal, 2022a, 2022b). Maleic anhydride (MA), the third most widely used anhydride globally, contains conjugated maleic acyl groups that modify the enzyme by replacing the positively charged amino group of the lysine molecule with a carboxyl group (Mozhaev, Melik-Nubarov, Šikšnis, & Martinek, 2009). For example, modifying amylase with MA has been reported to significantly improved its stability (Nwagu, Aoyagi, Okolo, Moneke, & Yoshida, 2020). A study on pineapple protease involving poly maleic anhydride also revealed an increase in thermal stability and surfactant tolerance (Xue, Wu, Branford-White, Ning, Nie, & Zhu, 2010). Acylation of casein hydrolysate using MA and encapsulation of curcumin was found provide better physicochemical stability (Pan, Li, Meng, Zhang, 2021).

MA has not previously been used to chemically modify XY to improve its catalytic activity and stability. Therefore, the present study aims to modify XY with MA then investigate the thermal stability, pH tolerance, and organic tolerance of the modified enzyme. Ultraviolet–visible (UV–vis) spectroscopy, fluorescence spectroscopy, circular dichroism (CD) spectroscopy and Fourier transform infrared (FT-IR) spectroscopy were used to study the structural changes in the enzyme and the altered catalytic properties associated with such changes. The clarification of fruits using MA-XY will then be investigated to determine its industrial potential. The development of an efficient, new, and practical method for improving the catalytic performance of XY would be of great significance for its wider application in the food industry.

2. Materials and methods

2.1. Materials

Xylanase and MA (99%) were purchased from Aladdin Biochemical Technology Co. (Shanghai, China), xylan (90%), xylose (99%), and 3,5-dinitrosalicylic acid (DNS) from Yuanye Biotechnology Co. Ltd. (Shanghai, China), and methanol, acetonitrile, and dimethylsulfoxide (DMSO) (99%, HPLC) from Sigma Aldrich Trading Co. Ltd., (Shanghai, China). The sodium dihydrogen phosphate and disodium hydrogen phosphate were analytically pure. All reagents used were of analytical, HPLC, or biological grade.

2.2. Purification of xylanase

A solution of XY (5 mg/mL) was prepared using buffer, and the suspension was centrifuged for 0.5 h at 4 °C for 4000 g. The supernatant containing the enzyme was dialyzed at 4 °C (with a molecular weight cut-off of 10 *kDa*) for 12 h to remove any excess salt ions. Afterwards, the purified enzyme was stored in the dark at 4 °C.

2.3. Measurement of xylanase activity

The catalytic activity of XY was measured as described by Mostafa *et al.* (Mostafa, El Aty, Hassan, & Awad, 2019). Five hundred μL of 1% beechwood xylan was mixed with 500 μL of the enzyme solution at 50 °C for 30 min. One mL of DNS was added to the reactants to terminate the reaction which was then boiled for 5 min and immediately cooled to room temperature. The absorbance of the solution was then measured at 540 nm by UV–vis spectroscopy. The enzymatic activity of XY was expressed as its ability to degrade xylan into reducing sugars per unit of time.

2.4. Measurement of enzymatic properties

2.4.1. Effect of adding MA on xylanase enzymatic activity

Briefly, 25 mg of XY were dissolved in 20 mL of phosphate buffer (0.2 M, pH 8.0) then 40, 60, 80, 100, and 120 mg of MA were added. The mixtures were stirred and kept at 14 °C for 4 h. To obtain the maleic anhydride-modified enzyme solution (MA-XY), the mixtures were dialyzed for 12 h. The enzyme activities of MA-XY and XY were measured as method *2.3*, then the relative enzyme activities were calculated.

2.4.2. Measurement of thermostability

The thermostability of XY and MA-XY was determined at 60 °C by measuring the activity every 20 min from 0 to 100 min. The enzyme activities of MA-XY and XY were measured as method *2.3*. The initial enzyme activity was used as the control. The relative activities of XY and MA-XY were then calculated.

2.4.3. Measurement of pH stability

The XY and MA-XY enzyme solutions (seven solutions each) were precisely adjusted to a pH of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. The pH stability was determined by incubating of XY and MA-XY for 30 min at 30 °C. The enzyme activities of MA-XY and XY were measured as method *2.3*. The respective relative enzyme activities of 100% were defined as the highest enzyme activity of XY and MA-XY.

2.4.4. Measurement of storage stability

The MA-XY and XY were stored at 4 °C in a phosphate buffer (0.2 M, pH 7.0) for 35 d and their activity was determined every 7 d. The enzyme activities of MA-XY and XY were measured as method *2.3*. The respective relative activities of XY and MA-XY on day 0 of storage were regarded as 100%.

2.4.5. Modified xylanase organic tolerance assay

2.4.5.1. Measurement of methanol tolerance. The methanol tolerance was determined by incubating XY and MA-XY with final methanol concentrations between 0% and 50%. Each mixture was evaporated at 30 °C for 30 min. The enzyme activities of MA-XY and XY were measured as method *2.3*. The enzyme activity for incubation at a methanol concentration of 0% was used as the control. The relative activities of the XY and MA-XY solutions were then calculated.

2.4.5.2. Measurement of acetonitrile tolerance. The acetonitrile tolerance was estimated by incubating XY and MA-XY with final acetonitrile between 0% and 25% at 30 °C for 30 min. he enzyme activities of MA-XY and XY were measured as method *2.3*. The enzyme activity for incubation without acetonitrile concentration was taken as control. The relative activity of XY and MA-XY were calculated.

2.4.5.3. Measurement of DMSO tolerance. The tolerance of XY and MA-XY to DMSO was evaluated by incubating XY and MA-XY with final DMSO concentrations between 0% and 50% at 30 °C for 30 min. he enzyme activities of MA-XY and XY were measured as method *2.3*. The

enzyme activity for incubation at a DMSO concentration of 0% was used as the control. The relative activities of XY and MA-XY were then calculated.

2.4.6. Measurement of xylanase kinetics

To determine the kinetic values of XY and MA-XY, their enzymatic activities were measured at different concentrations (6, 9, 12, 15, 18, 21, 24, 27, and 30 mg/mL) of beechwood oligosaccharide substrate (S). The activity was determined at the optimum reaction pH and temperature conditions determined during previous experiments. The kinetic parameters, the Michaelis constants (K_m, mg/mL) and maximum reaction velocity (V_{max}, /min), were calculated using the Lineweaver-Burk double inverse method by plotting (1/V and 1/S).

2.5. Measurement of spectra

2.5.1. Uv–vis spectroscopy

The XY and MA-XY solutions were examined by UV–vis spectroscopy using a UV-1900i spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The sample measurement solutions were prepared by dissolving a sample (200 μg/mL) in 0.2 M PBS at pH 7.0. Three identical samples were scanned at 200–400 nm using a slit width of 5 nm.

2.5.2. Fluorescence spectroscopy

The XY and MA-XY solutions were measured by fluorescence spectroscopy using an F-7000 fluorescence spectrometer (Hitachi Corporation, Kyoto, Japan). The sample measurement solutions were prepared by dissolving a sample (200 μg/mL) in 0.2 M PBS at pH 7.0. The scanning wavelength range was 300–500 nm on three identical samples using an excitation wavelength of 280 nm.

2.5.3. Circular dichroism (CD) spectroscopy

The CD spectroscopic data was obtained using a Chirascan CD spectrometer (Applied Photophysics, London, UK). The sample measurement solutions were prepared by dissolving a sample (400 μg/mL) in 0.2 M PBS at pH 7.0. The scanning speed was 5 nm/s, and the wavelength range 190–260 nm. Three identical samples were measured.

2.5.4. Fourier transform infrared (FT-IR) spectroscopy

The FT-IR spectra were determined using a Nicolet iS20 Fourier transform infrared spectrometer (Bruker, Berlin, Germany). The samples were prepared using the KBr compression method, and measurements were made over a wavelength range of 400–4000 cm^{-1}, with 32 scans at a resolution of 8 cm^{-1} and a scan interval of 2 cm^{-1}. Three identical samples were measured.

2.6. MA-XY and XY for juice clarification determination

As described by Rosmine *et al.* (Rosmine, Sainjan, Silvester, Alikkunju, & Varghese, 2017) described how oranges, mangoes, pineapples, bananas, pears, and citruses were peeled then pulped. In the present study, the fruits pulps and water were blended using a blender to obtain a smooth pureé, which was incubated with XY and MA-XY (1:1) at 55 °C for 4 h. After boiling for 5 min to inactivate the enzyme, the sample were centrifuged at 4000 g for 10 min, and the supernatant was collected. The control group used for samples without xylanase. The optical density was measured at 650 nm, and the reducing sugar content estimated using the DNS method.

2.7. Statistical analysis

The experiments were repeated three times, and the results expressed as the mean ± SD. Origin 2017 software (Origin Lab Corporation, Washington DC., USA) was used to create graphs, and SPSS version 23 (IBM Corporation, Armonk, NY, USA) to analyze the data. The significance level for differences between mean values was set at $P < 0.05$.

3. Results and discussion

3.1. Effect of adding MA on xylanase activity

The catalytic activity of the unmodified enzyme, XY, was found to be 26 U/mg in the present study. The present study investigated the effect of MA on the catalytic activity of XY, which was modified by adding different amounts of MA then determined the relative catalytic activities of these modified enzymes, MA-XY. Fig. 1 shows that the relative enzyme activity of MA-XY increased gradually as the amount of MA added increased when the amount of MA added was low. The lysine residues on the XY molecule were not modified sufficiently so that the relative enzyme activity of MA-XY was low. The maximum relative enzyme activity of MA-XY was 154.32% when 100 mg MA were added to 25 mg of XY. However, when the amount of MA added reached a certain value, the relative enzyme activity of MA-XY decreased. This occurred because, at a certain point, adding MA led to a relatively high degree of modification, causing unfavorable modifications of the XY catalytic function, steric hindrance, and a reduction in catalytic activity. The change in the position of the carboxyl group of the acid anhydride during the modification of the lysine residue also led to different changes in the enzyme activity. Similarly, Xue *et al.* used poly maleic anhydride to modify pineapple protease and found that the higher the concentration of the modifier, the higher the degree of modification, resulting in a greater number of modified lysine residues (Xue, Wu, Branford-White, Ning, Nie, & Zhu, 2010). Some studied have reported that enzyme activity was increased by modification with an acid anhydride (Szabó, Kotormán, Laczkó, & Simon, 2009).

3.2. Analysis of thermal stability

The thermal stability of enzymes is an essential factor in its industrial applications. To evaluate the thermal stability of enzymes, the catalytic activity of MA-XY and XY were determined after incubation at high temperatures (60 °C) for 0 to 100 min. The relative enzyme activity changes are shown in Fig. 2A. At 60℃, the relative enzyme activities of MA-XY and XY decreased rapidly to 26.67% and 10.56%, respectively, after incubation for 20 min. When incubated for 100 min, XY lost almost all its original catalytic activity but the relative enzymatic activity of MA-XY was 13.34%. The thermal stability profiles of MA-XY were broader than those of XY. MA modifies the amino group of XY, and the lysine residues mainly alter the positive charge of the protein. This positive charge can neutralize the inner charge of the polypeptide and reduce the repulsive force (Quintanilla-Guerrero et al., 2008), therefore, making MA-XY more stable. It has also been reported that MA modifies the protein molecule by replacing the positively charged amino group of the lysine molecule with a carboxyl group, thus changing the charge properties of the enzyme surface (Mozhaev, Melik-Nubarov, Šikšnis, & Martinek, 2009). At the same time, modifying XY by MA improves hydrophilicity, thus reducing the damage to the enzyme at high temperatures. Similarly, Nwagu *et al.* used acid anhydride to modify an enzyme chemically and found that its resistance to high temperatures was enhanced (Nwagu, Aoyagi, Okolo, Moneke, & Yoshida, 2020). Primarily, the modifier compound surrounds the spatial conformation of the enzyme and acts as a protective barrier around the protein (Chen, Zhang, Dong, Liu, & Sun, 2020), thus preventing damage to the enzyme protein molecule and providing protection to cysteine, thereby improving enzyme activity.

3.3. Analysis of pH tolerance

After MA modification, the tolerance assay of MA-XY to pH was determined using buffer solutions with a pH ranging from 3.0 to 9.0. Fig. 2B shows that XY activity was greatly affected by pH, with its

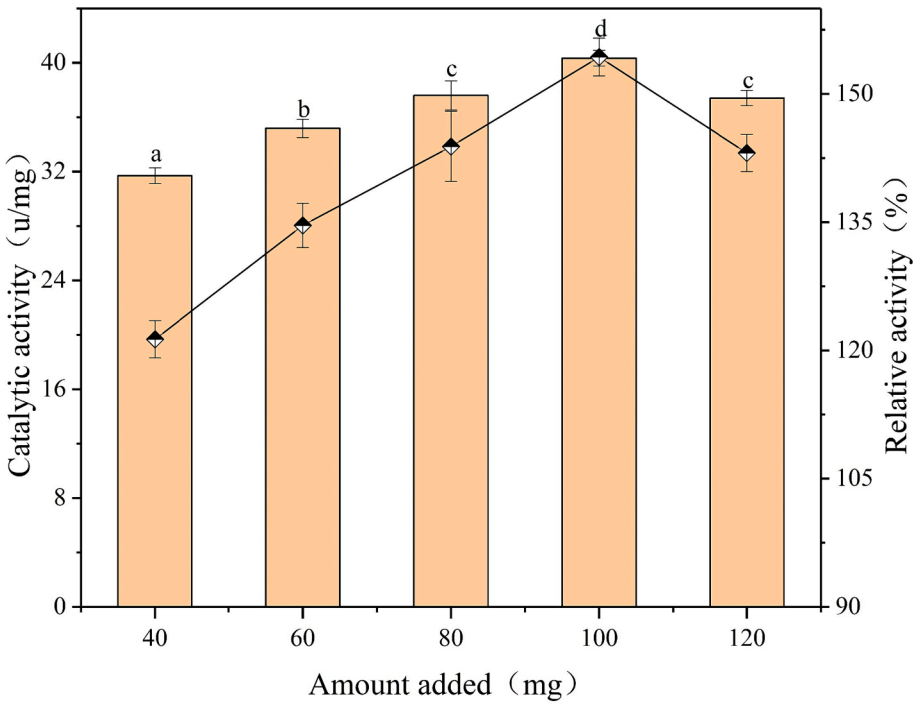

Fig. 1. The effect of maleic anhydride addition on xylanase.

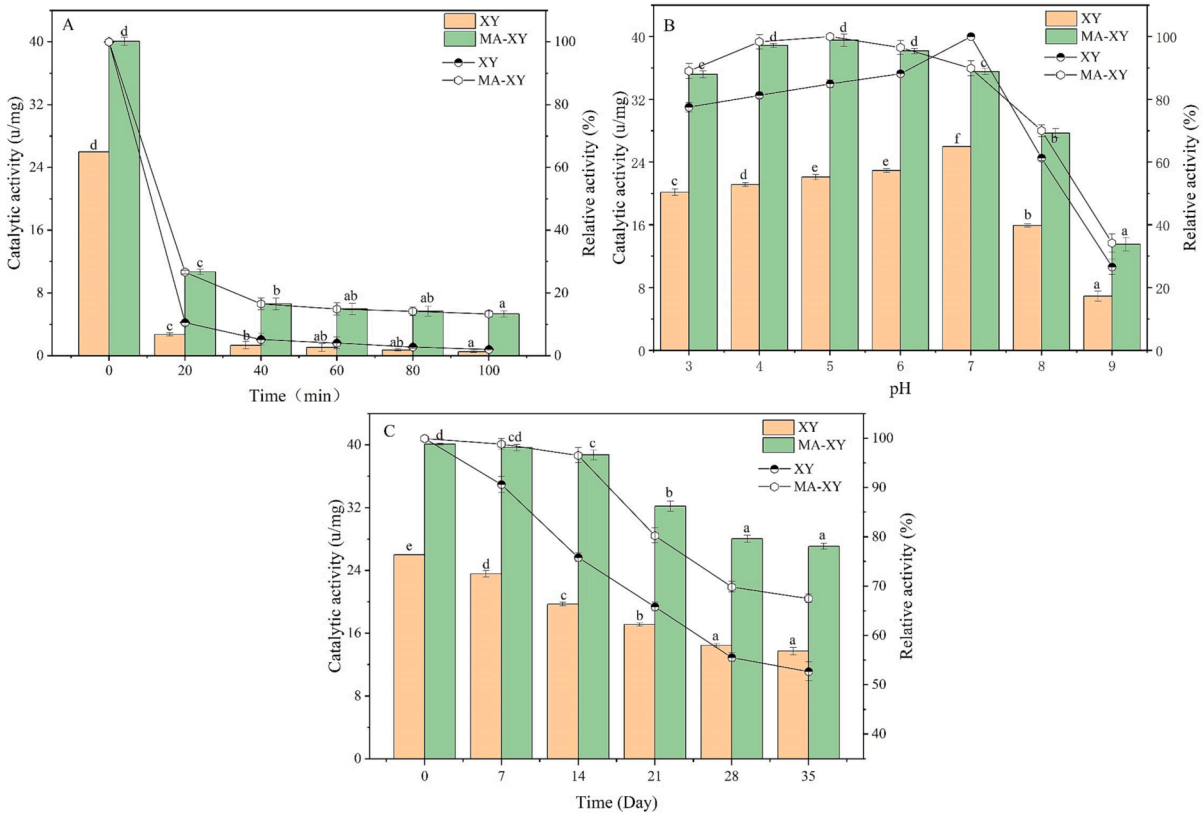

Fig. 2. (A) Thermal Stability of XY and MA-XY. (B) pH stability of XY and MA-XY. (C) Storage stability of XY and MA-XY.

maximum value at pH 7.0, while MA-XY was 39.54 U/mg which was 1.18 times more active than that of XY (33.51 U/mg) at a pH 5.0. These results indicated that adding MA could effectively improve the pH tolerance of XY. Similarly, the modification of amylase with MA has been reported to decreased its optimum pH by one unit, a similar trend to that observed in the present study (Nwagu, Aoyagi, Okolo, Moneke, & Yoshida, 2020). This was mainly caused by the negative charge of the MA carboxyl group, which had replaced the positive charge of the lysine group of the enzyme protein during the acylation reaction, resulting in a decrease in pH (Shaw, Schneider, Bilgier, Kaufman, & Whitesides,

2008). Yandri *et al.* (Yandri, Apriyanti, Suhartati, & H Ad I, 2016) obtained similar results when modifying α-amylase using different concentrations of citraconic anhydride with a 0.5unit reduction in its optimum pH.

3.4. Analysis of storage stability

The XY and MA-XY solutions were stored at 4 °C, and their enzyme activity was measured every 7 d. Fig. 2C shows that the relative activities of MA-XY and XY gradually decreased with increasing storage time. Over almost 21 d, the relative activity of XY rapidly decreased to 65.85%. However, the enzyme activity of MA-XY was still 1.26 times greater than that of XY, and the relative enzyme activity of XY had rapidly decreased to 55.58 % by the 28th day of storage, at which that of MA-XY was 67.52 %. The MA-XY activity was 1.28 times that of XY on the 35th day of storage.

3.5. Analysis of organic solvent tolerance

When XY is exposed to organic solvents, it easily loses its activity, particularly with solvents having a relatively strong polarity. The water layer on the surface of the XY molecule can easily be removed thus reducing or losing its catalytic activity. High concentrations of organic solvents destroy the hydrophobic core of the enzyme protein, which protects the hydrophobic core of the protein (Wang et al., 2019). Improving the tolerance of XY to organic solvents, particularly strongly polar solvents, is crucial for increasing the industrial applications of XY. Since some substrates of XY have different miscibility in different organic solvents, this study mainly investigated the effects of different organic reagents on XY and MA-XY.

3.5.1. Methanol tolerance analysis

Studies have shown that strong hydrogen bonds can inactivate enzymes: as methanol is a protic solvent, strong hydrogen bonds can be formed, and a certain amount of water is required to preserve the catalytic activity of enzymes. However, in polar solvents, the water available for the enzyme molecule is deprived, with the relative enzyme activity decreases (Wu, Zhang, & He, 2001). The changes in the tolerance changes of MA-XY and XY to different concentrations of methanol (Fig. 3A) showed that MA-XY has a better tolerance then XY. At a methanol concentration of 10%, the relative enzyme activity of MA-XY decreased slightly but was still 6.58% higher than that of XY. At a methanol concentration of 50%, the MA-XY activity was approximately 61.78% of its initial value, whereas that of XY decreased significantly more to be 50.26 %.

3.5.2. Acetonitrile tolerance analysis

The enzyme activities of the MA-XY and XY were determined at different acetonitrile concentrations, after 30 min of exposure. The changes in hydrolytic activity are shown in Fig. 3B. MA-XY was more resistant to acetonitrile than XY but was still affected by acetonitrile to a certain extent. As the concentration of acetonitrile increased, the hydrolytic activity of MA-XY gradually decreased, yet remained higher than that of XY. The enzyme activity of MA-XY was reduced to 23.31% at an acetonitrile concentration of 25%, but was still 1.31 times greater than that of XY. One study has shown that after modifying papain with monocarboxylic and dicarboxylic acids, its tolerance to organic solvents was significantly enhanced (Szabó, Kotormán, Laczkó, & Simon, 2009), with its catalytic activity in water-soluble and buffered organic solvents significantly enhanced, and its stability improved to a certain extent. Zou *et al.* (Zou, Yan, Xia, Zhang, & Adesanya 2020) used functional ionic

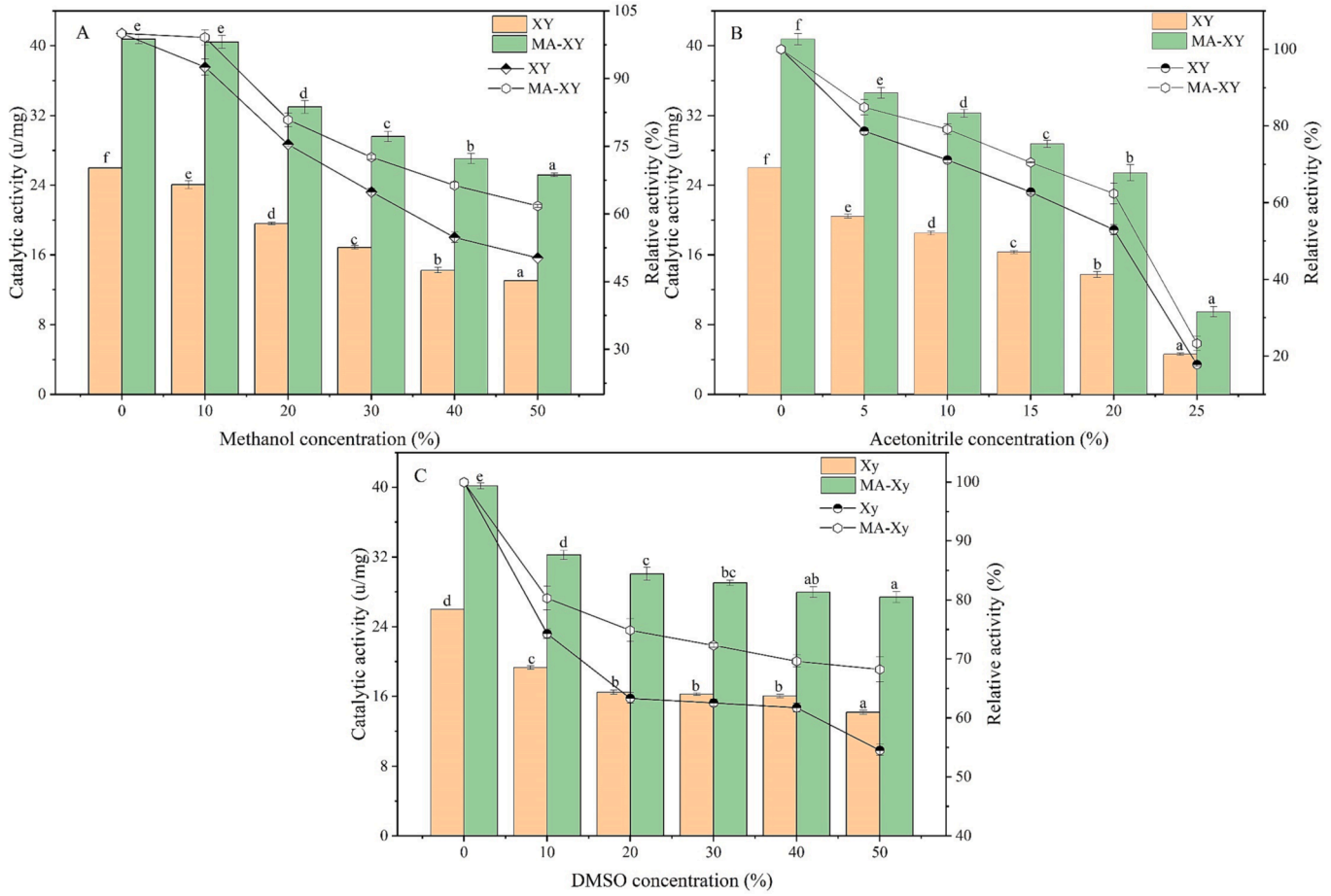

Fig. 3. (A) Methanol tolerance of XY and MA-XY. (B) Acetonitrile tolerance of XY and MA-XY. (C) DMSO tolerance of XY and MA-XY.

liquids to modify lipase and showed that the modified enzyme molecules had better DMSO tolerance than methanol tolerance. This may be due to organic solvents of different polarities, which alter the chemical and physical properties of the catalytic environment of the enzyme.

3.5.3. Analysis of DMSO tolerance analysis

The tolerance of MA-XY and XY to different concentrations of DMSO, at 30 °C, was determined by measuring enzyme activity over 30 min. The activity of MA-XY and XY decreased to varying degrees with increasing DMSO concentrations. MA-XY activity decreased rapidly after treatment with DMSO at 20%, but was still 1.18 times greater than that of XY (Fig. 3C). Regarding the tolerance of MA-XY and XY to DMSO at a concentration of 50%, XY only retained 54.57% of its initial activity, while that of MA-XY retained 68.27%. Therefore, MA-XY was more stable in DMSO. Li *et al.* (Li, Zhang, Li, Huang, & Hu, 2015) modified lipase using polyethylene glycol, and reported the its tolerance to organic solvents and thermal stability were improved. Similarly, the tolerance to different organic solvents varied after modification of laccase using acid anhydride, with acetonitrile being the most stimulating to the modified enzyme and DMSO the least stimulating, in agreement with the results of the present study. This may be because the acyl group provided by the anhydride increases the hydrophobicity of the enzyme surface (Danait-Nabar, & Singhal, 2022a, 2022b).

3.6. Analysis of reaction kinetic parameters

The catalytic efficiency of an enzyme is related to the rate constant of the enzyme reaction, the maximum rate (V_{max}), and the Michaelis constant (K_m). Fig. 4 shows that the kinetic constant of XY changed to a certain extent. The K_m value of MA-XY was 30.23 mg/mL, which was less than that of XY at 40.63 mg/mL, with V_{max} increasing from 0.29 to 0.31 min^{-1}. This decrease in K_m indicated that the affinity between the enzyme and the substrate had improved in the case of MA-XY. The increase in V_{max} indicated that the reaction rate of catalysis of the substrate had increased so that is the catalytic efficiency of MA-XY was better. Therefore, compared with XY, MA-XY exhibited a better catalytic efficiency and substrate affinity, mainly because of the changes in its spatial structure after modification with MA, which changed the molecular environment of the enzyme and improved the catalytic efficiency of the enzyme.

3.7. Spectroscopic analysis

3.7.1. Ultraviolet–visible spectroscopy

The UV–vis spectra of MA-XY and XY were measured to determine their secondary structures. Tryptophan, phenylalanine, and tyrosine, which are aromatic amino acids, can stimulate the absorption of UV–vis light by proteins via the excitation of electrons. The absorption spectra of the chromophores of MA-XY changed to some extent. The UV–vis spectra of MA-XY and XY are shown in the Fig. 5A. After MA modification, the UV–vis absorption peak changed to a certain extent.

The near-UV–vis spectral region (250–350 nm) of proteins depends mainly on the type of aromatic amino acids, its environmental properties, and the aerial configuration of the protein, and can therefore be used to detect changes in the tertiary structure of proteins (Kelly, & Price, 2019). MA-XY and XY exhibited the same UV–vis spectrum, with a positive band at approximately 285 nm, and the UV–vis absorption peak being enhanced after modification, indicating a smaller reorientation of the aromatic compounds. This also indicated that the MA-XY tertiary structure had been significantly affected by MA, possibly because of changes in the microenvironment of the aromatic and heterocyclic group amino acid residues caused by the binding of the enzyme protein to MA (Xiong, Gao, Zheng, & Deng, 2011). The near-UV–vis spectrum of XY exhibited two positive spectral bands at 269 nm and 287 nm, mainly because of the presence of tyrosine. Similarly, Song *et al.* (Song, Yao, Liu, Zhou, Xiong, & Ji, 2005) used acid anhydride to modify horseradish peroxidase and found that the tertiary structure around the aryl hydroxylate residue of the modified enzyme had been altered, but the α-helix content had increased while promoting an increase in organotypic stability.

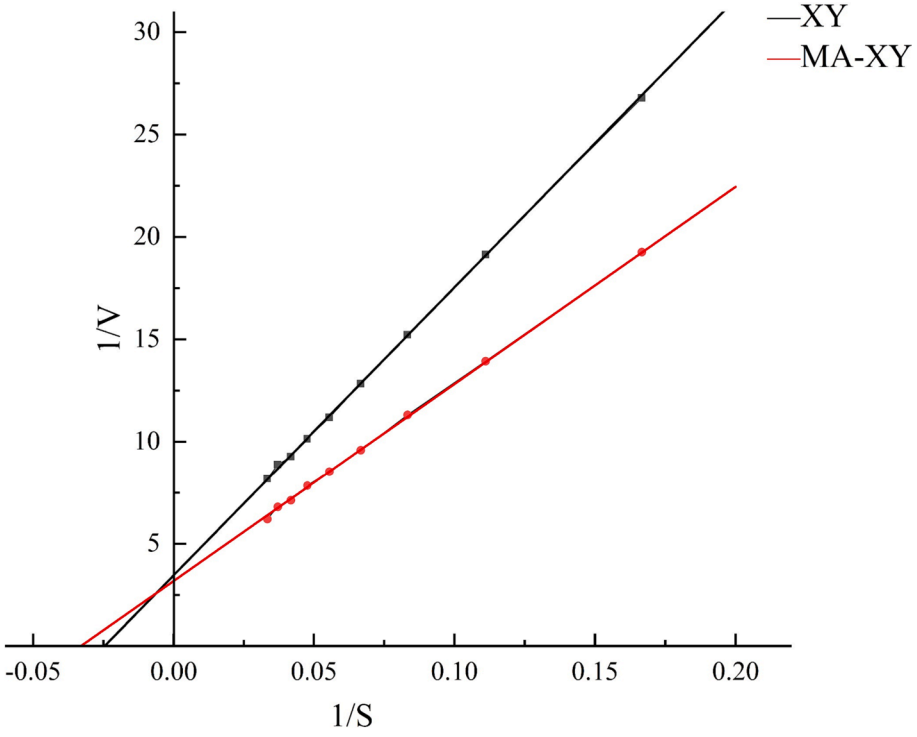

Fig. 4. Kinetics of XY and MA-XY.

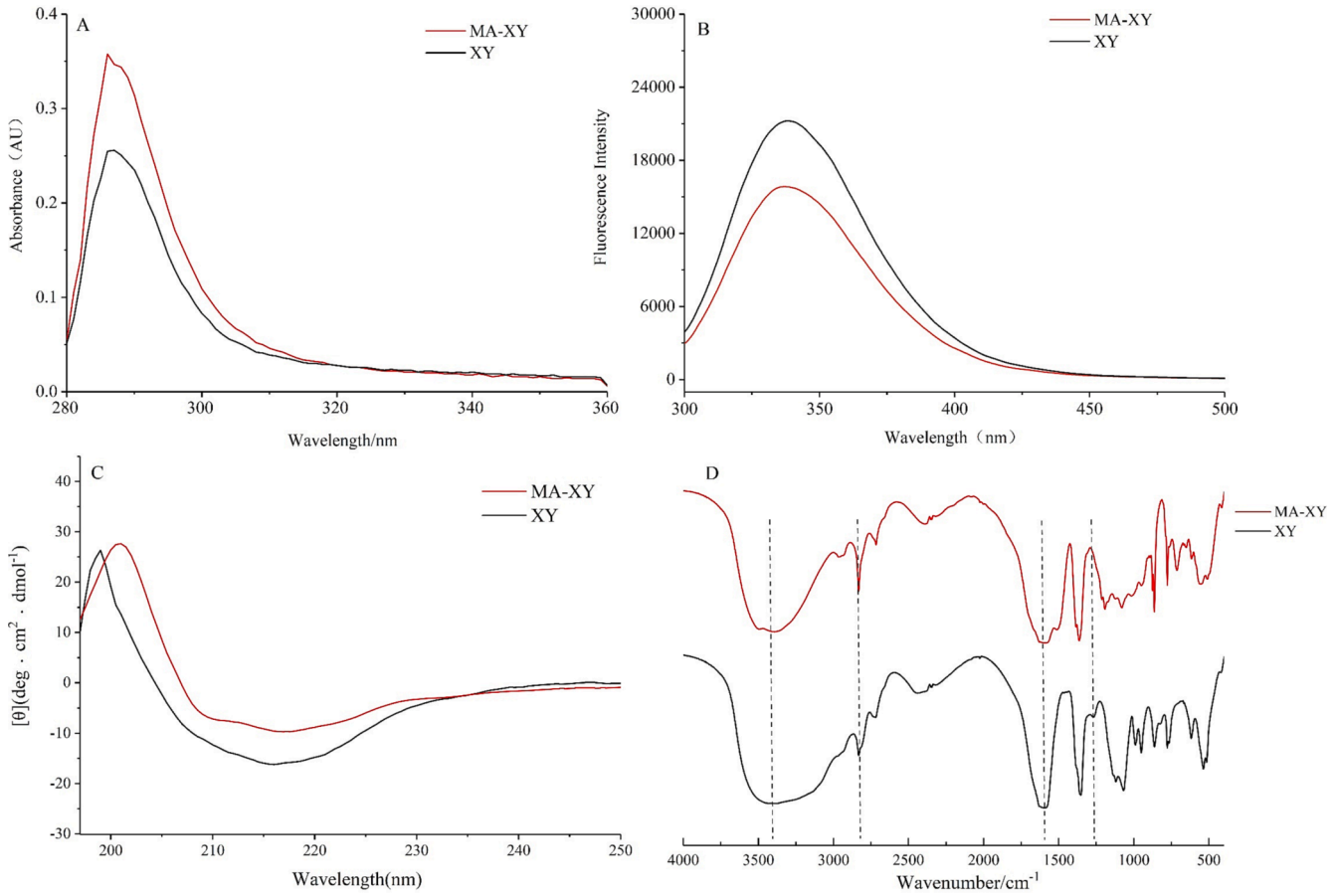

Fig. 5. (A) Ultraviolet–Visible spectroscopy of XY and MA-XY. (B) Fluorescence intensity of XY and MA-XY. (C) Circular Dichroism spectroscopy of XY and MA-XY. (D) Fourier Transform Infrared Spectroscopy of XY and MA-XY.

3.7.2. *Fluorescence spectroscopy*

Fluorescence spectroscopy is considered a powerful tool for studying protein conformational changes in proteins, and tryptophan, as an excitation group for fluorophores, is helpful for the sensitive analysis of the tertiary structure of MA-XY. Proteins that form additional hydrogen bonds or disulfide bonds, or experience changes in the local environment, can cause conformational changes. In the present study, the structural changes in MA-XY were studied using fluorescence spectroscopy (Fig. 5B).

The emission spectrum in the 335–350 nm range indicated that tryptophan had been encapsulated in the hydrophobic water of the protein, which is located in a region of relatively low polarity. The 350–353 nm range also indicates if tryptophan has been exposed to water (Liu & Gou, 2008). After MA modification, the red-shift of the tryptophan fluorescence emission peak of MA-XY at approximately 340 nm indicated that the interaction between MA and XY not only had led to some changes in the microenvironment of tryptophan but also indicated the dotted group near the tryptophan residue (Vivian, & Callis, 2001). The fluorescence intensity of MA-XY in the present study was significantly less than that of XY because of the occurrence of acylation and a significant decrease in the emission intensity of the tryptophan spectrum. This suggested that the mutual binding ability between some proteins in MA-XY had been weakened, resulting in certain changes in the protein structure and a decrease in the water solubility of tryptophan, leading to a burst reaction and a decrease in fluorescence intensity. Nwagu *et al.* (Nwagu, Aoyagi, Okolo, Moneke, & Yoshida, 2020) modified amylase with citraconic anhydride and maleic anhydride and observed a slight decrease in the emission intensity of the fluorescence spectrum. Similarly, Szabo *et al.* (Szabó, Kotormán, Laczkó, & Simon, 2009) found that XY modified with MA and citraconic anhydride decreased the tryptophan spectrum.

3.7.3. *Circular dichroism spectroscopy*

The secondary and tertiary structures of XY and its catalytic activity are closely related, with its structure being affected by various factors. In the present study, CD was used to determine the secondary structures of the enzyme proteins, including structural changes (Fig. 5C).

XY exhibited its lowest CD signal at 214.5 nm and was red-shifted after MA modification, indicating that MA had influenced the structure of XY. It was also found that the intensity of the negative shoulder peak was reduced after MA modification, especially for the peak at 200–210 nm, indicating a reduction in β-folding or irregular curling (Yang, Wu, & Martinez, 1986). The calculations showed that the β-fold and irregular curl of MA-XY shifted to the α-helix and β-turn angles. This was mainly caused by the introduction of the maleate anion group through acylation, which increased electrostatic repulsion and spatial site resistance, leading to conformational changes in the protein.

3.7.4. *Fourier transform infrared spectroscopy*

FT-IR is widely used as a practical and fast technique for studying the structure and interactions of proteins. When functional groups interact at the molecular level, the functional groups and secondary structures of enzymes can be identified by changes in their FT-IR spectra. These include changes in the position or intensity of the absorption bands and the appearance of new bands (Martins, Cerqueira, Bourbon, Pinheiro, Souza, & Vicente, 2012).

FT-IR spectroscopy was performed on MA-XY and XY (Fig. 5D and Table 1). The secondary structure of MA-XY had been significantly altered by acylation modification: amide I, amide II, and amide III bands were observed at 1600, 1433, and 1294 cm^{-1}, respectively, related to

Table 1
Secondary structure of XY and MA-XY.

	XY	MA-XY
Helix	32.10%	39.70%
Beta	32.30%	0.00%
Turn	22.20%	54.10%
Random	13.30%	6.20%

C═0 stretching, N—H deformation, and C—N stretching, respectively. The absorption band of XY was relatively wide compared with that of MA-XY. Its absorption intensity at 1600 cm^{-1} was higher than that of XY, mainly because of the reaction of MA with amino groups and thus the formation of l-amides. This explains the change in complexation condensation in the carbonyl region after MA modification. There was also a relationship between the 2927 cm^{-1} spectral band and the anti-symmetric stretching of C—H in the CH_2 and CH_3 groups (Pirestani, Nasirpour, Keramat, Desobry, & Jasniewski, 2018).

The bands in the 3700–3200 cm^{-1} and 1100–1000 cm^{-1} regions represent the stretching vibration of the absorption peak of the C—O bond. The intensity and number of vibrations in the 1000 cm^{-1} band of MA-XY were significantly higher than those observed for XY, mainly because of the acylated modified enzyme, which had substantially changed the secondary structure of the enzyme as demonstrated by the change in its relative absorption peak. The bands at 1500–800 cm^{-1} were mainly attributable to C—H deformation, C—O, and C—C stretching. This also indicated the formation of covalent bonds via the acylation reaction between MA and XY.

3.8. Analysis of xylanase on clarified fruit juices

Juice viscosity and turbidity are caused by carbohydrate-activated polymers such as pectin, hemicellulose, and lignin (Kumar, Nagar, Mittal, Garg, & Gupta, 2014). Table 2 shows that the reducing sugar content and clarity of the juice were improved after treatment with MA-XY because xylanase induces the degradation of xylan, which reduces juice viscosity and thus its transparency. MA-XY also helped to increase the reducing sugar content of the juice by releasing reducing sugars through the enzymatic digestion of xylan. The results showed that the absorbance values of all fruit juices decreased to different degrees with the most significant decreases occurring in mango, in particular, a decrease in its turbidity, and an increase in its reducing sugar content. The present study showed that MA-XY performed well at clarifying juice compared with XY. This result was consistent with its biochemical properties, suggesting that XY with MA modification has excellent potential for juice clarification. Studies in which fruits were clarified by using enzymes to obtain fruit juice have also been reported (Wang, Owusu-Fordjour, Xu, Ding, & Gu, 2020). Similarly, Streptomyces xylanase was used to clarify orange juice and pineapple juice by 20.87% and 27.89%, respectively (Rosmine, Sainjan, Silvester, Alikkunju, & Varghese, 2017).

4. Conclusions

In the present study, MA was used to modify XY, with an appropriate amount of MA promoting enzyme activity to a certain extent. The enzymatic activity of MA-XY was studied, and showed that the thermal, pH, and storage stabilities of the modified enzyme were better than that those of XY. Kinetic studies and spectral analyses also proved that MA-XY had a stronger ability to bind substrates and a more stable structure than XY. The present study has provided a practical theoretical basis for the promoting the application of XY in the food industry. It is worth noting that MA-XY had effectively improved juice clarification, by breaking down the cloudiness of juice caused by large polysaccharides. Therefore, MA-XY has excellent potential to improve the quality of fruit juice through its use as a clarifying agent as well as its wider application in the food industry.

Table 2
Clarification of some fruit juice by using MA-XY.

Fruits	% increase in the reducing sugar amount		% increase clarification	
	XY	MA-XY	XY	MA-XY
Orange	13.25 ± 0.12^{a}	18.68 ± 0.11^{b}	25.67 ± 0.14^{a}	36.85 ± 0.21^{b}
Mango	15.22 ± 0.03^{a}	25.57 ± 0.02^{b}	32.19 ± 0.18^{a}	46.87 ± 0.31^{b}
Pineapple	2.98 ± 0.02^{a}	5.48 ± 0.06^{b}	10.23 ± 0.16^{a}	16.79 ± 0.09^{b}
Banana	4.19 ± 0.05^{a}	11.46 ± 0.11^{b}	14.46 ± 0.08^{a}	22.57 ± 0.13^{b}
Pear	3.62 ± 0.14^{a}	10.96 ± 0.11^{b}	10.79 ± 0.08^{a}	22.22 ± 0.17^{b}
Citrus	4.81 ± 0.08^{a}	7.23 ± 0.10^{b}	16.12 ± 0.14^{a}	18.54 ± 0.16^{b}

Different letters in the same column indicate significant differences ($P < 0.05$).

CRediT authorship contribution statement

Yang Zhao: Methodology, Data curation, Formal analysis, Writing – original draft, Software. **Luyue Zhang:** Writing – review & editing, Validation. **Shiyu Zhang:** Data curation. **Xing Zheng:** Supervision. **Mingzhu Zheng:** Project administration. **Jingsheng Liu:** Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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