Electronic Supplementary Material (ESI) for Green Chemistry.

Supporting Information

Engineering of Skin Fibre Opening enzyme for Sulphide-free Leather Beam House Operation through Xenobiology

Suryalakshmi Pandurangan[ae], Ilamaran Meganathan[a], Sriram Ragavan[b], Kamini Numbi Ramudu[a], Easwaramoorthi Shanmugam[c], Ganesh Shanmugam[d], and Niraikulam Ayyadurai[ae*]

a. Department of Biochemistry and Biotechnology, Council of Scientific and Industrial Research – Central Leather Research Institute (CSIR-CLRI)

b. Department of Crystallography and Biophysics, University of Madras

c. Department of Inorganic and Physical Chemistry, CSIR-CLRI, Chennai, India

d. Department of Organic and Bioorganic Chemistry, CSIR-CLRI, Chennai, India

e. Academy of Scientific and Innovative Research, CLRI Campus, Chennai, India

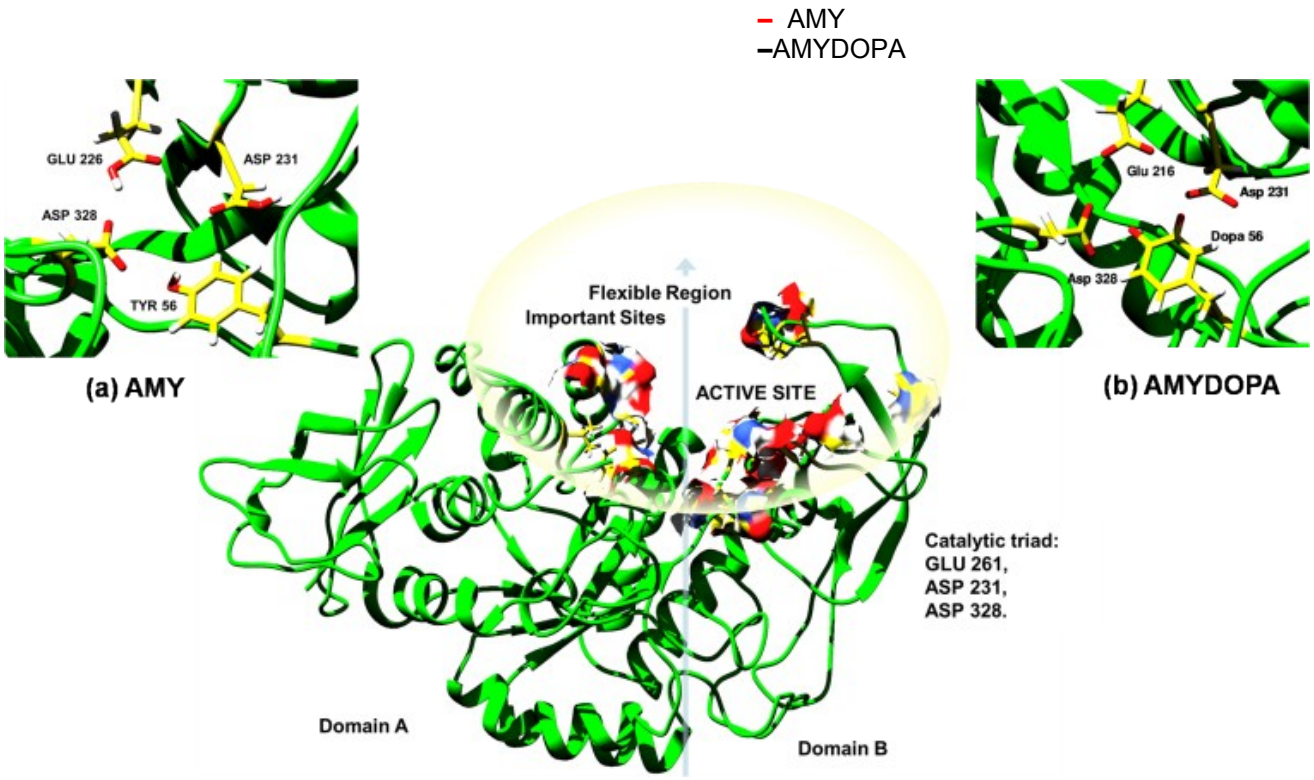


Figure S1: Archetypal representations of the active site pocket of AMY with catalytic residues **(a)** Tyrosine 56 in the active site, **(b)** DOPA 56 in the active site.

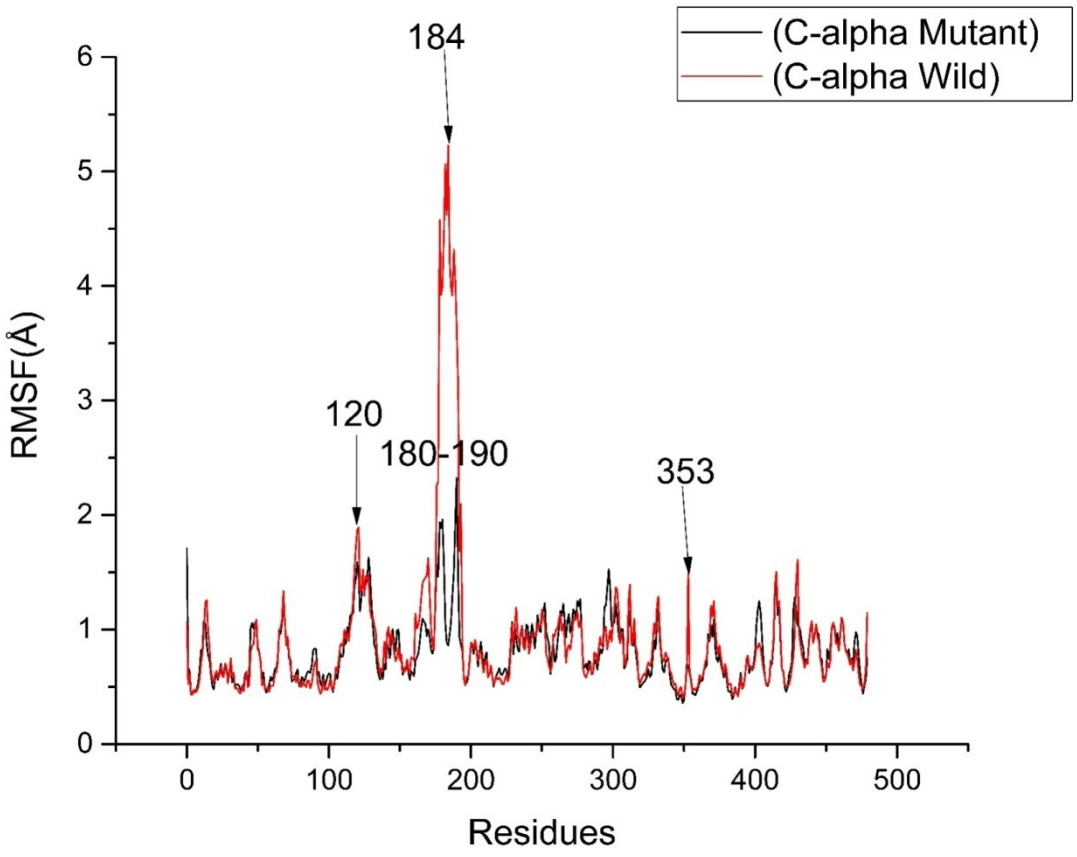
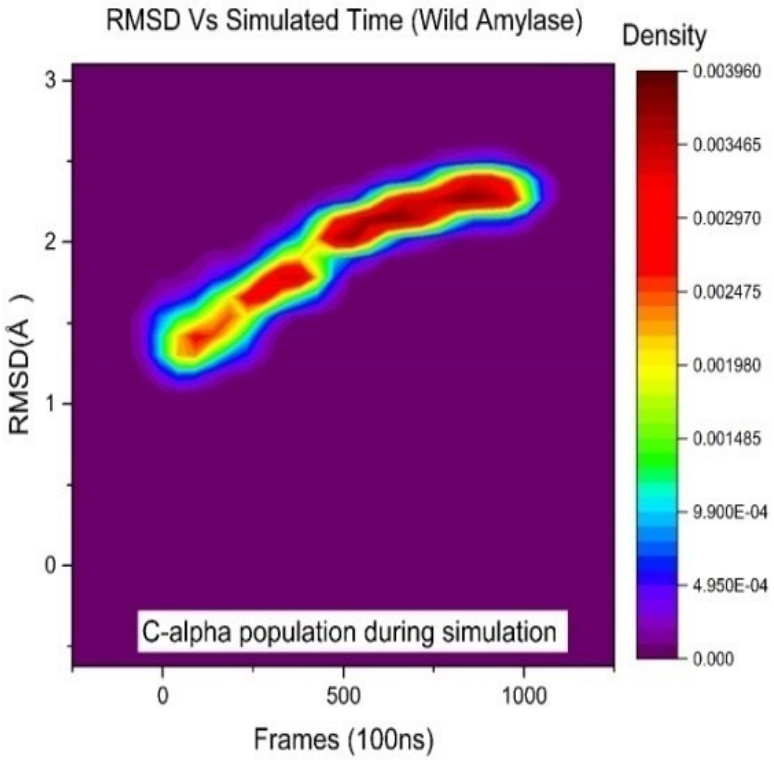


Figure S2: Residual fluctuation plot with respected to the simulated time of both AMY and AMYDOPA variant

(a)



(b)

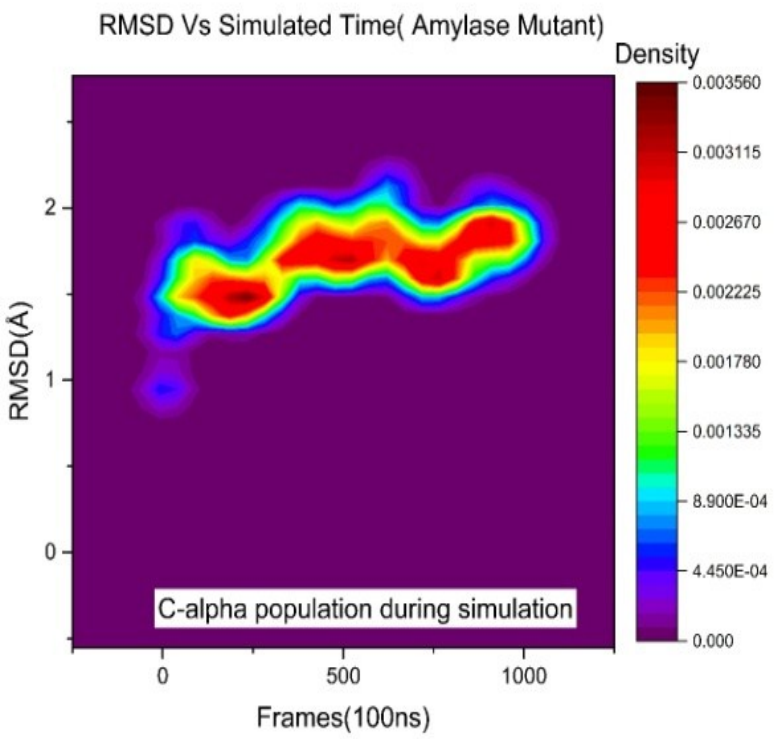
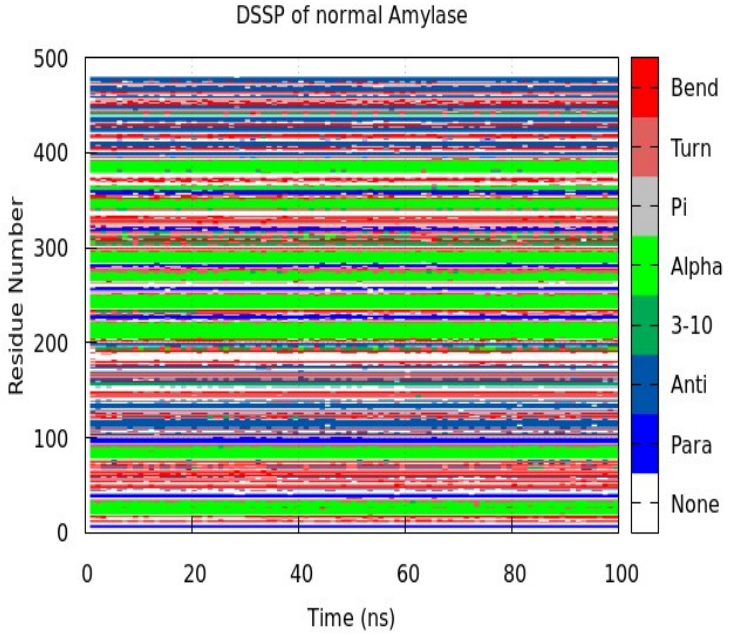


Figure S3: C-alpha RMSD PCA estimates of residual deviation from the crystallographic derived structure. (a) RMSD PCA of AMY (b) RMSD PCA of AMYDOPA.



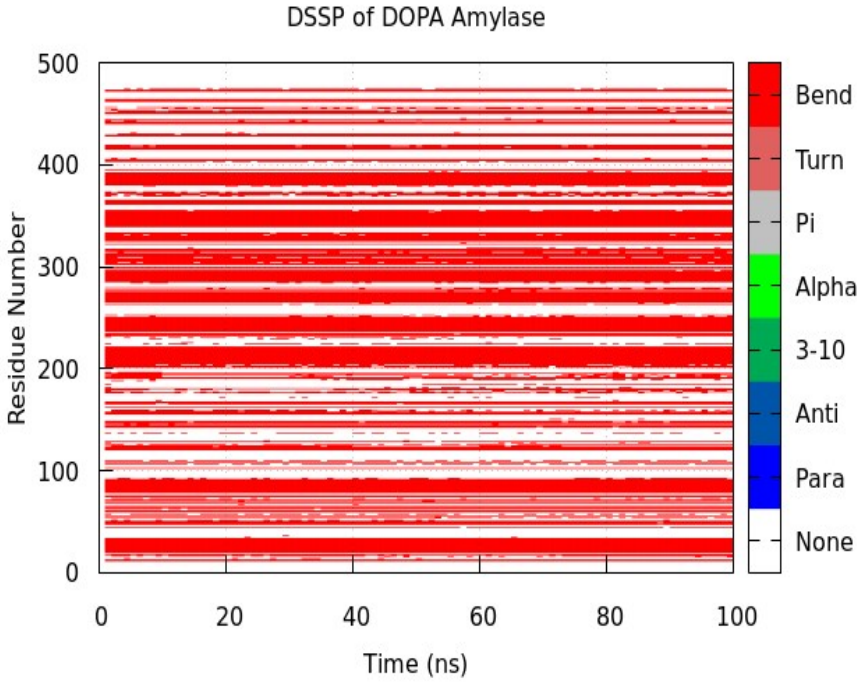


Figure S4: DSSP plot of AMY and AMYDOPA.

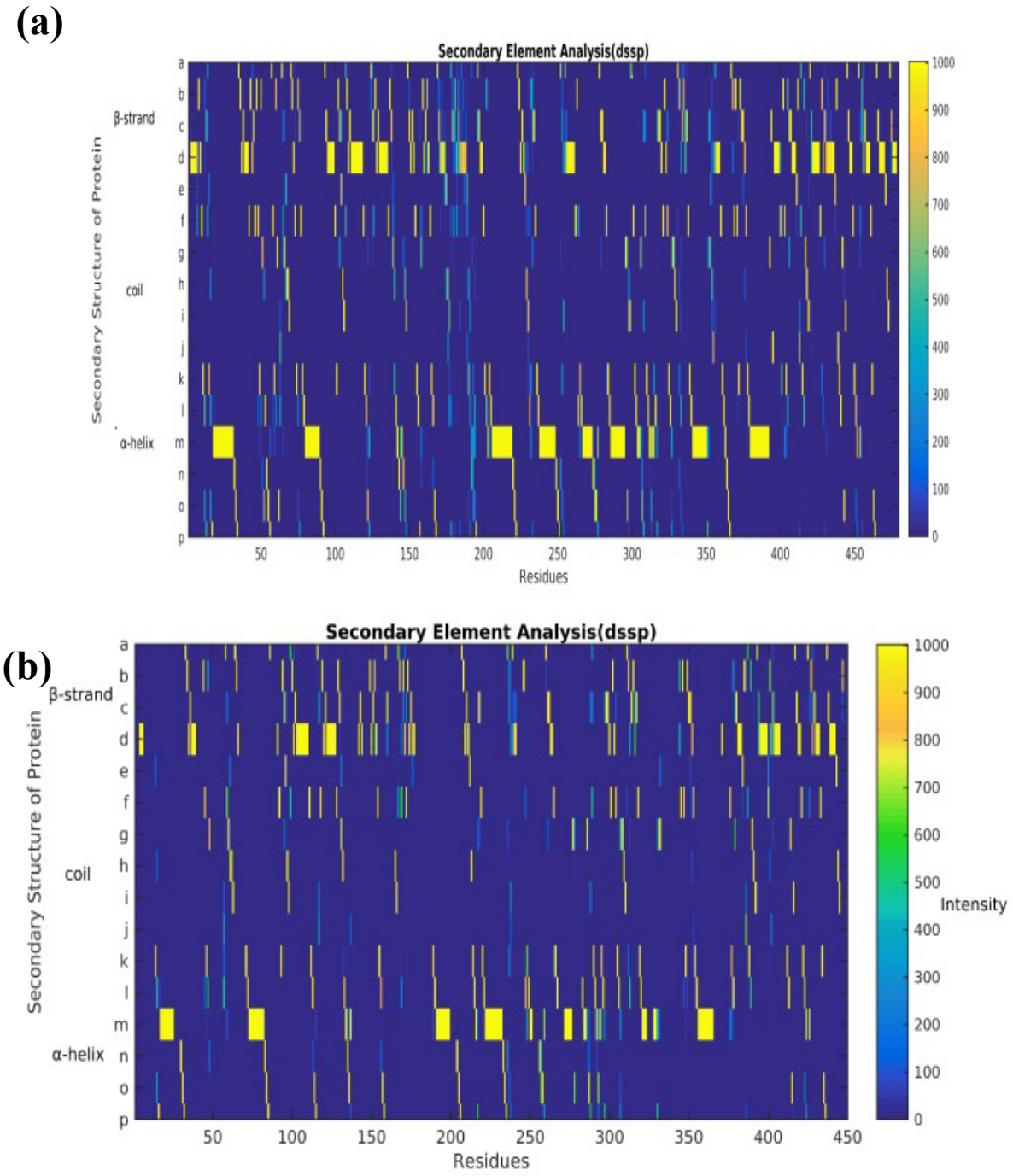


Figure S5: **The p**air wise dynamic secondary structure plot of overall AMY (a) and AMYDOPA (b) where a-p represents a difference in backbone dihedral angle.

Table T1: Table representing the docking score of various substrates with AMY and AMYDOPA.

Substrate	**Docking score**	
	AMY	**AMYDOPA**
Chondroitin sulfate	-12.445	-16.119
Dextrin	-15.080	-14.982
Starch	-10.789	-11.472
Melibiose	-10.790	-10.360
Lactose	-12.702	-12.201

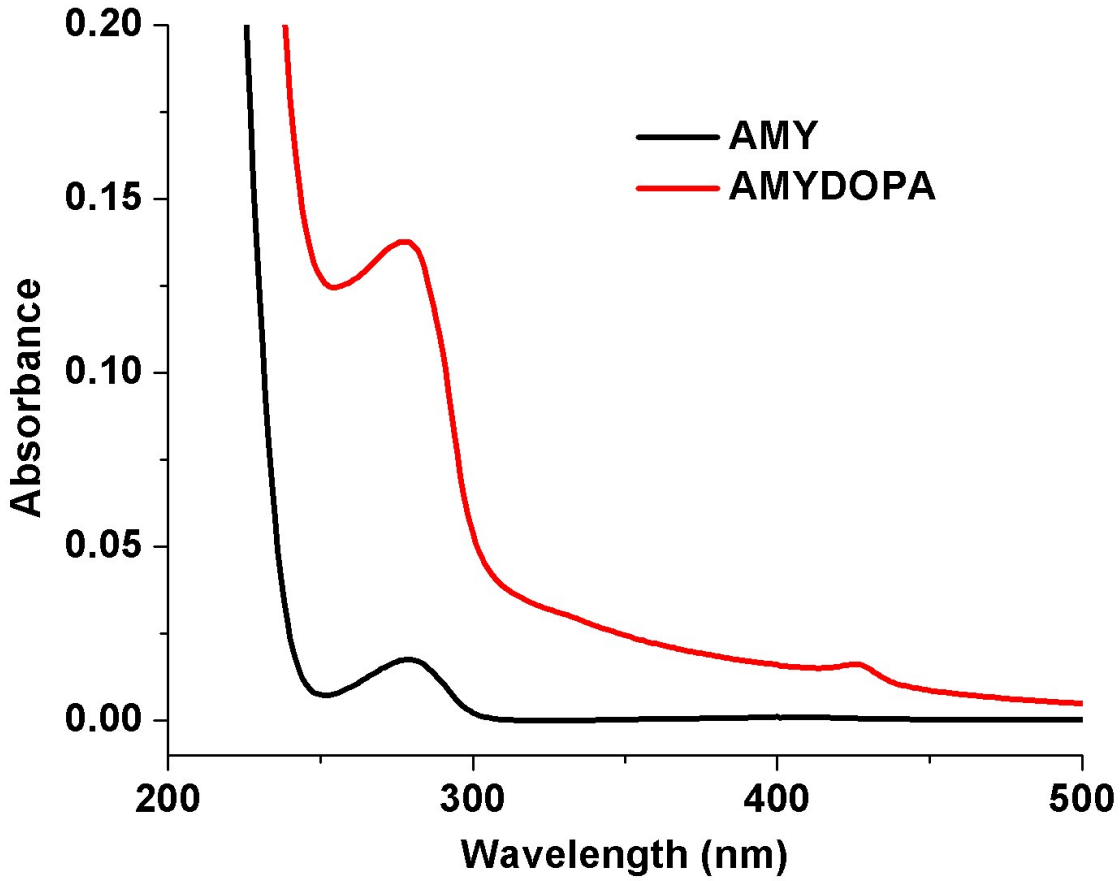


Figure S6: UV-Visible spectrum of AMY and AMYDOPA.

Over expression of AMY and AMYDOPA:

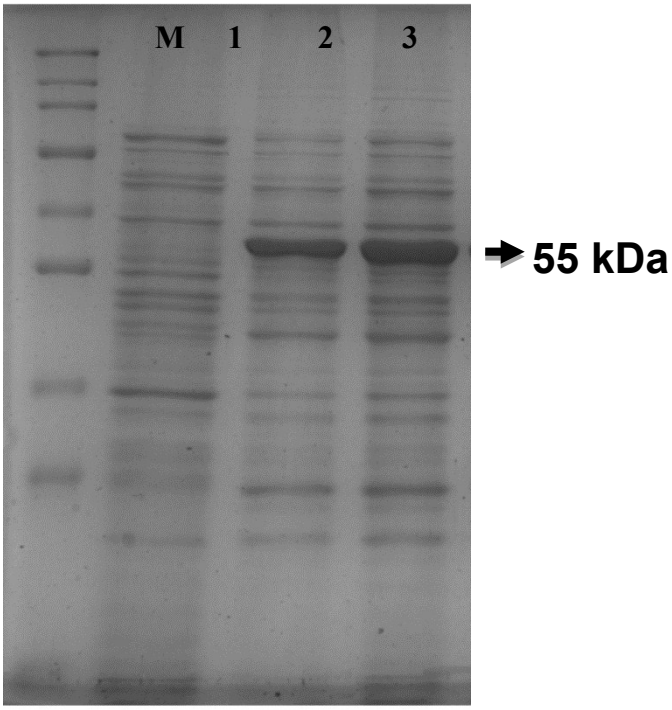


Figure S7: Expression of AMY and AMYDOPA in *E. coli* JW2581 were confirmed in SDS-PAGE. Lane1: *E. coli* pQE-80L, 2: *E. coli* pQE-80L-AMY, 3: *E. coli* pQE-80L-AMY-DOPA

Redox cyclin staining

Preliminary confirmation of DOPA in the protein sample was done by redox cyclin staining. The poly vinylidene difluoride membrane (PVDF) was activated by washing it three times and incubating it for 5 min in methanol. Residual methanol was removed by washing with Milli Q water. Proteins were spotted in activated PVDF membrane and stained with nitroblue tetrazolium (NBT) solution (2 M Potassium glyconate buffer, 0.25 mM NBT, pH 10.0) for 3 h in dark room. Further, the membrane was soaked in 0.16 M sodium borate solution overnight for visualization.

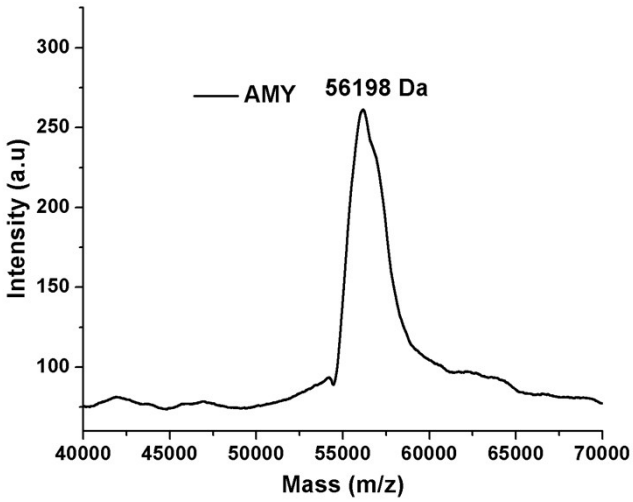
1 2 3 4

Figure S8: Redox cyclin staining of AMY and AMYDOPA; 1: Negative control (tyrosine), 2: Positive control (DOPA), 3: AMY, 4: AMYDOPA

Mass spectroscopy analysis

The mass difference between AMY and AMYDOPA were identified using Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) (Bruker Daltonics, Billerica, MA, USA) for the indirect confirmation of DOPA incorporation. The purified desalted proteins were spotted in MALDI-TOF target plate and was analysed in ultrafleXtreme^{TM} systems (Bruker Daltonics, Billerica, MA, USA) comprising next generation MALDI-TOF technology. The mass spectrometer was performed with positive reflectron mode and the spectra were acquired in the range of 40000 - 70000 m/z.

A



B

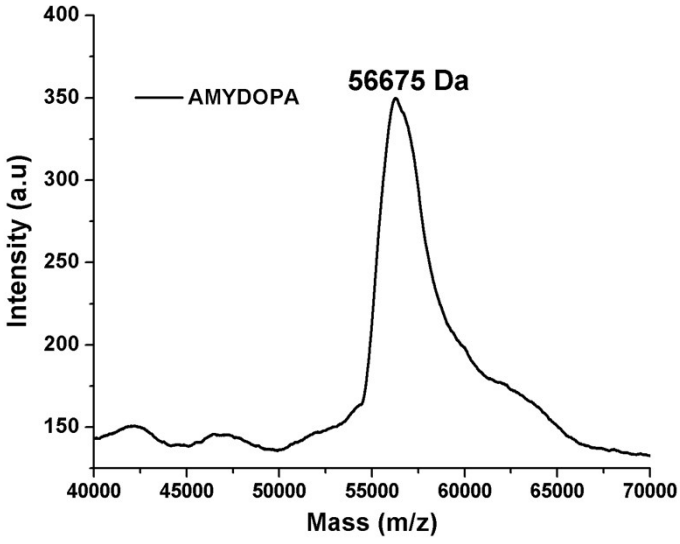


Figure S9: The total mass of AMY (A) and AMYDOPA (B) were analysed by MALDI-TOF using linear mode. Intact mass analysis of AMY and AMYDOPA is 56675 Da and 56198 Da respectively. Increased mass is indicating that 30 tyrosine were replaced with DOPA.

Circular Dichroism

The CD spectra were measured using JASCO J715 spectropolarimeter with a 1 mm rectangular quartz cell. The instrument was constantly purged with N_2 gas during CD measurements. All the CD spectra were measured between 190 and 250 nm with a scanning rate of 50 nm/min. The bandwidth, data pitch, and response time was set to as 1 nm, 0.5 nm and 1 s, respectively. The concentration of both AMY and AMYDOPA was 0.5 mg/ml. To obtain the baseline correction, the contribution of the buffer was subtracted from the sample CD spectrum. For thermal studies, the temperature of the cell holder was increased from 25 to 95 °C using a JascoPeltier temperature controlled accessory. In each desired temperature, the sample solution was incubated at 2 min before recording the CD spectrum.

Individual intensity measurement

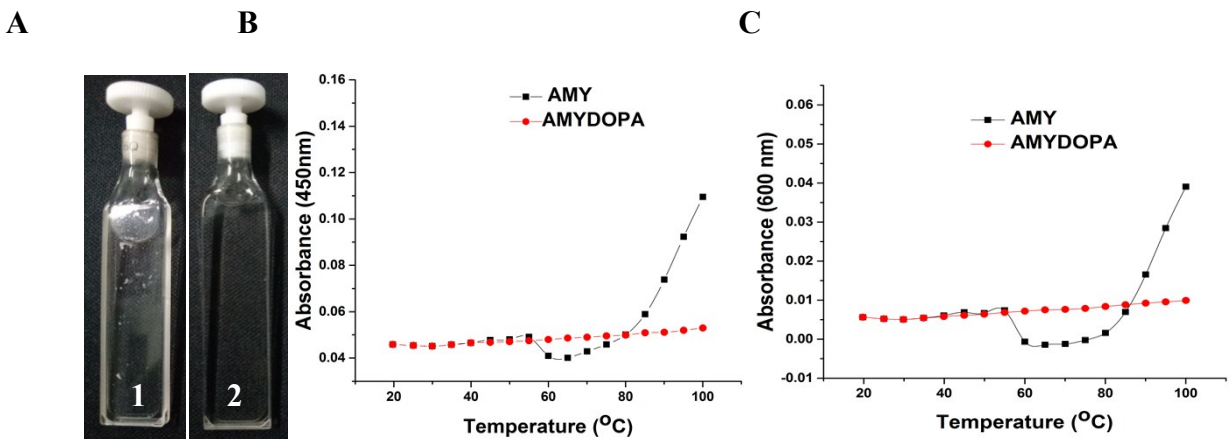


Figure S10: A. AMY (1) shows the aggregation when increasing the temperature above 80°C whereas AMYDOPA (2) is didn't show any aggregation when increasing the temperature. B, C. Individual absorbance intensity of AMY and AMYDOPA at 450 and 600 nm in different temperature. Absorbance intensity of AMY was increased after 80°C indicating its aggregation.

Fluorescence spectrum

Fluorescence spectra of AMY and AMYDOPA were measured to confirm the tertiary structure in terms of thermal stability. 1 mg/ml of each protein in 10 mM phosphate buffer (pH 7.4) were studied in different temperature ranging from 25–95 °C in a JASCO FP-777 Spectrofluorimeter (Jasco, Tokyo, Japan) equipped with a thermostat. The proteins were excited in 290 nm wavelengths under 500 V, 2.5 slit width with the scanning speed of 240 nm/min, and the fluorescent spectrum of both the proteins was recorded.

Physicochemical properties

Relative activity based on temperature and pH was measured to assess the difference in physicochemical properties of between AMY and AMYDOPA. The assay temperature and pH was varied to find the optimum enzymes performance condition. The assay was performed from 30 °C to 90 °C and a pH from 3 to 10.

Effect of various solvents

Residual activity of the enzymes was analyzed in the presence of water-miscible solvents and water immiscible solvents. Solvents such as Acetate, Ethylacetate, Hexane, Isopropanol, Methanol, DMSO, Acetonitrile, Butanol, and Ethylbeneze were used for this study. The enzymes were mixed with 50% of solvents for 1 hr in room temperature followed by the activity was measured under standard assay conditions. This residual activity was compared with the activity of enzymes without any solvents which also been stored in a similar condition like a test.

Residual activity:

The residual activity was measured in different temperature and pH to identify the enzyme stability. AMY and AMYDOPA were incubated in a different condition such as 30–40 °C and pH 3–10 for 1-4 h. After the incubation, the assay was performed under the standard condition to find its residual activity.

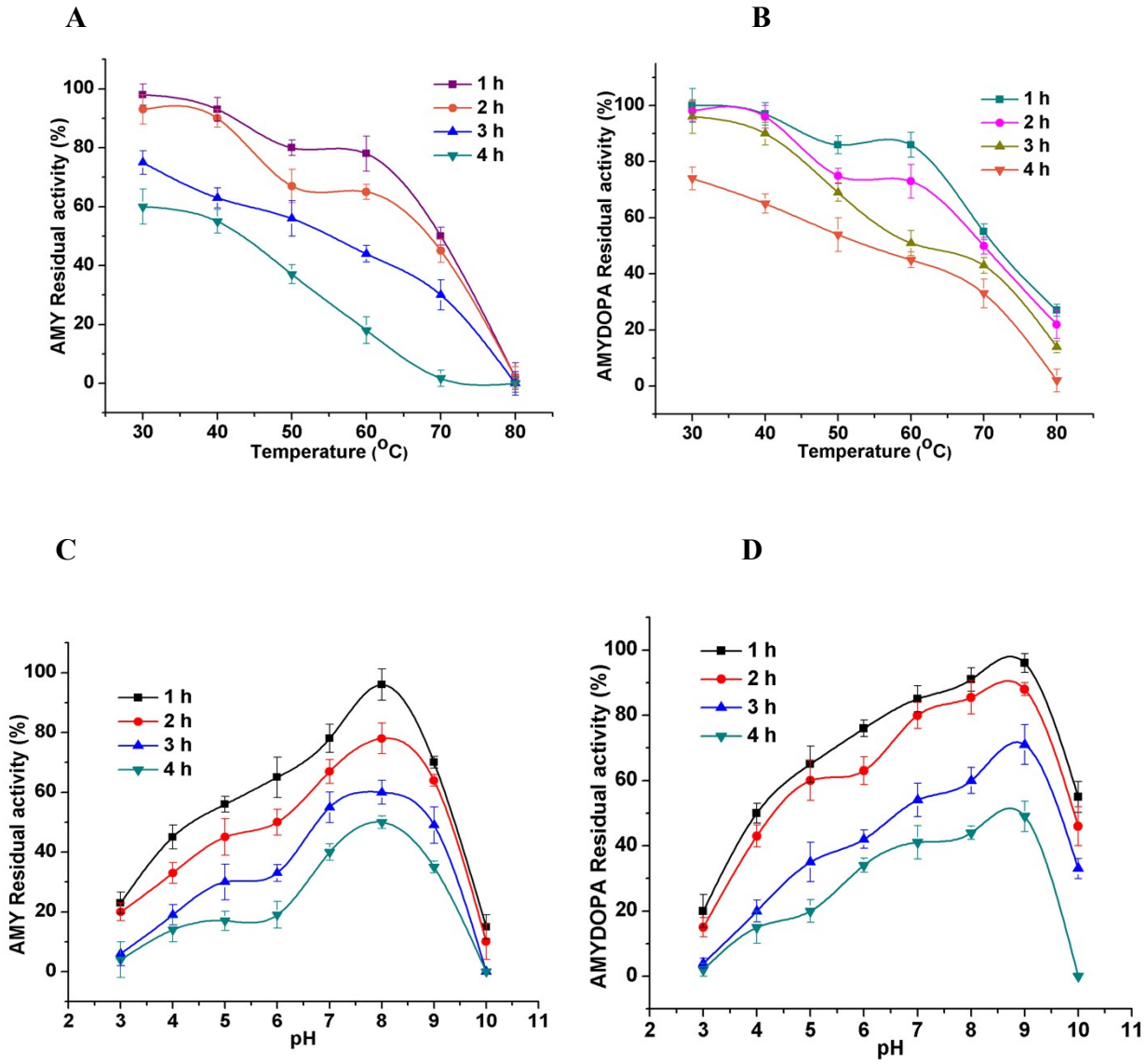


Figure S11: Residual activity of AMY and AMYDOPA in different temperature and pH for 1-4 h; A: Residual activity of AMY in different temperature, B: Residual activity of AMYDOPA in different temperature, C: Residual activity of AMY in different pH, D: Residual activity of AMYDOPA in different pH

Effect of various metal cations

To find the metal cofactors and inhibitors for AMY and AMYDOPA, relative activity assay was performed with different metal ions. 10 mM of different metal ions were added with the substrate to study its impact on the functional properties of enzymes. The assay was performed under standard assay condition using a substrate with metal ions.

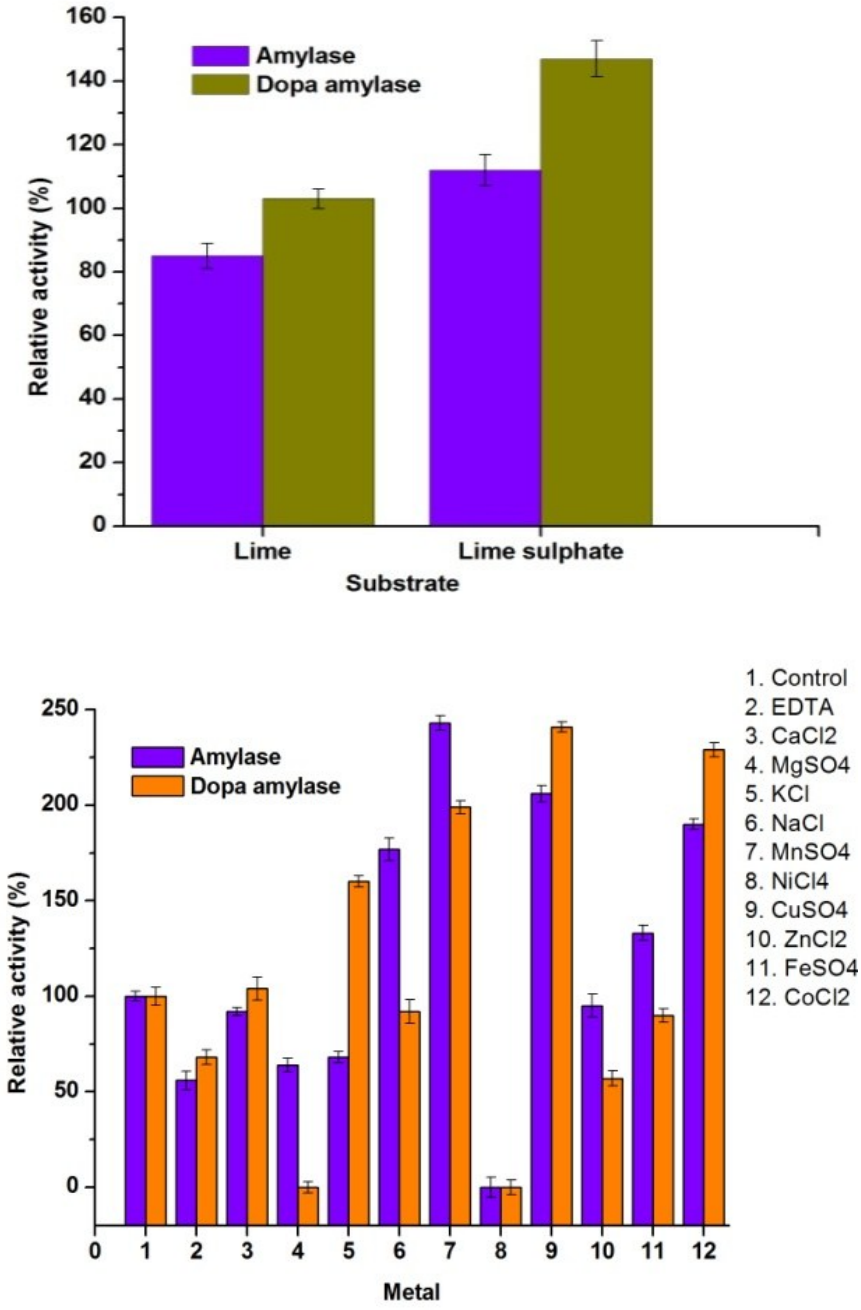


Figure S12: Relative activity of AMY and AMYDOPA in the presence of a chemical used in leather industries (lime and lime sulphate) (A) and in the presence of various metal ions (B).

FTIR analysis:

Equal concentration of purified enzymes and metals were incubated for 15 min at room temperature to study the interaction of enzyme and metal by FTIR analysis. Treated samples were coated on a CaF_2 plate and air dried. FTIR spectra were recorded on a Perkin Elmer spectrum TWO spectrometer at room temperature. FTIR spectra were collected at a resolution of 8 cm^{-1} with a total of 32 scans. All spectra were baseline corrected.

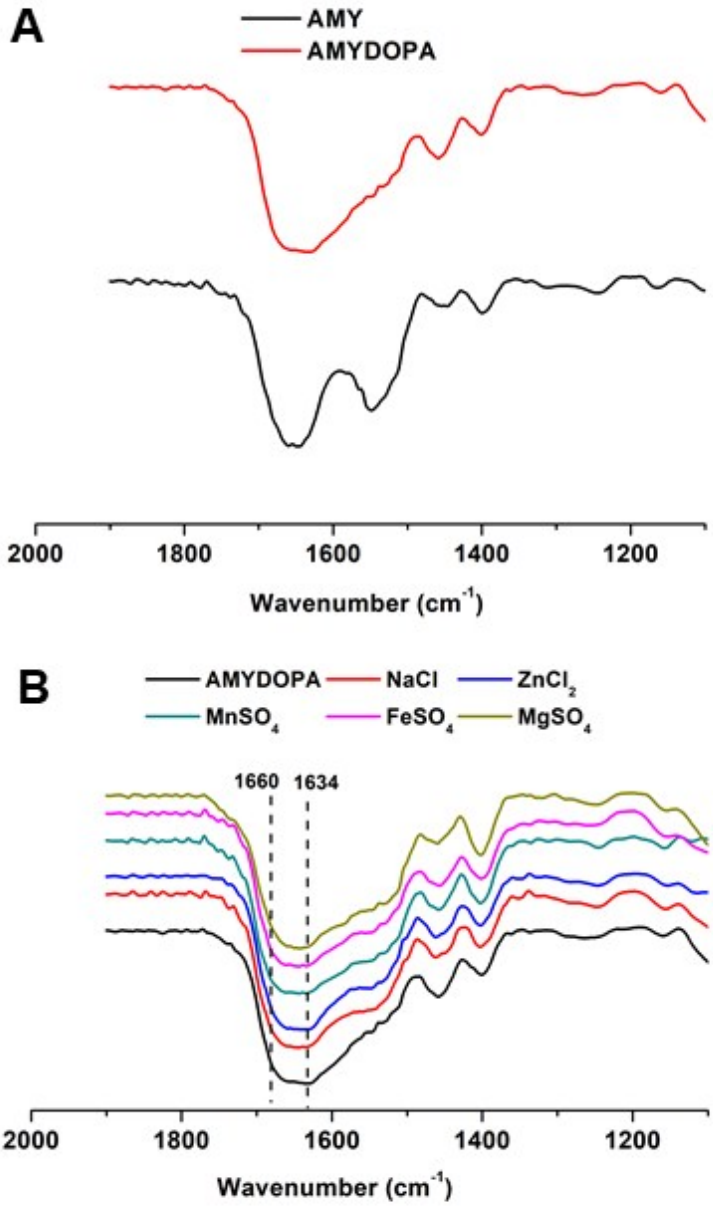


Figure S13: *(*A) FTIR spectra of AMY and AMYDOPA. (B) FTIR spectra of AMYDOPA in the absence and presence of different metal ions.

Relative activity of AMY and AMYDOPA in the water collected from tanneries:

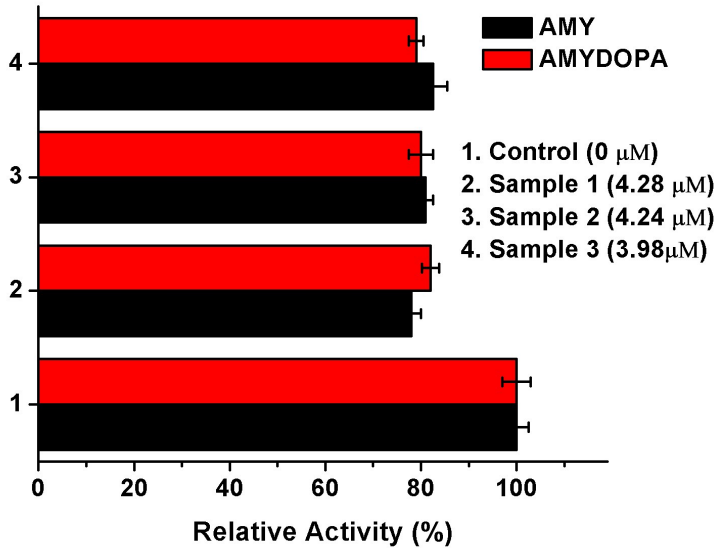


Figure S14: Relative activity of AMY and AMYDOPA were analyzed in three different tap water collected from the various location in Chennai, Tamilnadu, India. The available NaCl in water samples were analysed through inductively coupled plasma mass spectrometry. The concentration of NaCl identified as follow 4.28 μM (sample 1), 4.24 μM (sample 2) and 3.98 μM (sample 3) respectively.

Time-dependent product release from the substrate

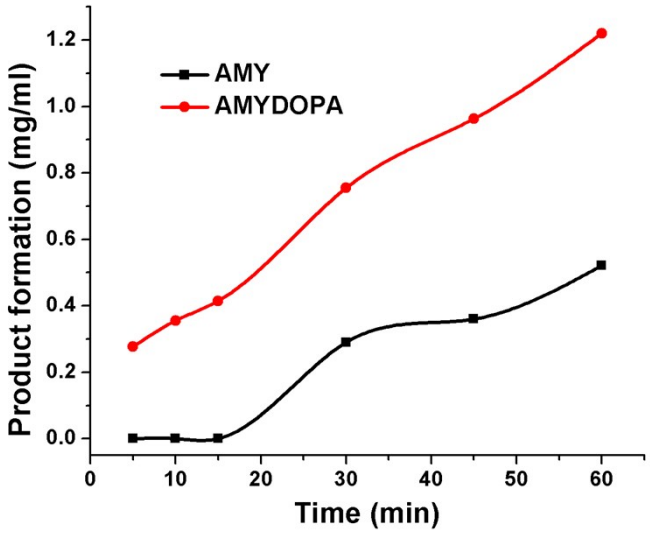


Figure S15: Time courses of product formation. 1 % of chondroitin sulfate was incubated with 1 U of enzymes at 37 °C for 60 min. Amount of monosaccharide released from the substrate was estimated at different time using colorimetric assay.

Histology studies

The AMY and AMYDOPA efficacy in the reduction of compact fibers in the skin was compared using Hematoxylin and Eosin staining (HE staining). For the differentiation of collagen fibers Masson's trichrome staining was used. The treated skin samples were taken from FDC and stored in 10% neutral buffered formalin for 3 days and skins embedded in paraffin. 5 µm thick sections were prepared using microtome and stained using HE and Masson's trichrome staining. The stained skin samples were viewed under a Nikon Eclipse E600 microscope.

Substrate specificity of enzymes

To study the substrate specificity of AMY and AMYDOPA performed using various substrate under the standard assay condition and different polysaccharides containing α-1,4-glycosidic linkage were prepared in 100 mM phosphate buffer (pH 6.5). The soluble potato starch was replaced with different polysaccharides such as lactose, melibiose, corn starch, maltodextrin and chondroitin sulfate for this study.

Enzyme kinetics

Steady-state kinetics study was performed using a different concentration of chondroitin sulfate varying from 0 to 1.6 mM. Enzyme activity was performed under standard assay condition. V_{max}(maximum rate of the reaction at saturating substrate concentration), K_M (Michaelis constant substrate concentration at which the reaction rate is half of V_{max}), and k_{cat} (Maximum number of substrate molecule convert into product per active site per unit of time) were calculated using Michaelis Menten non linear plot and were listed below.

Enzyme	V_{max} (U/mg/min)	K_M (M)	k_{cat} (min^{-1})
AMY	1.15	0.32	0.163
AMYDOPA	2.586	0.383	0.368

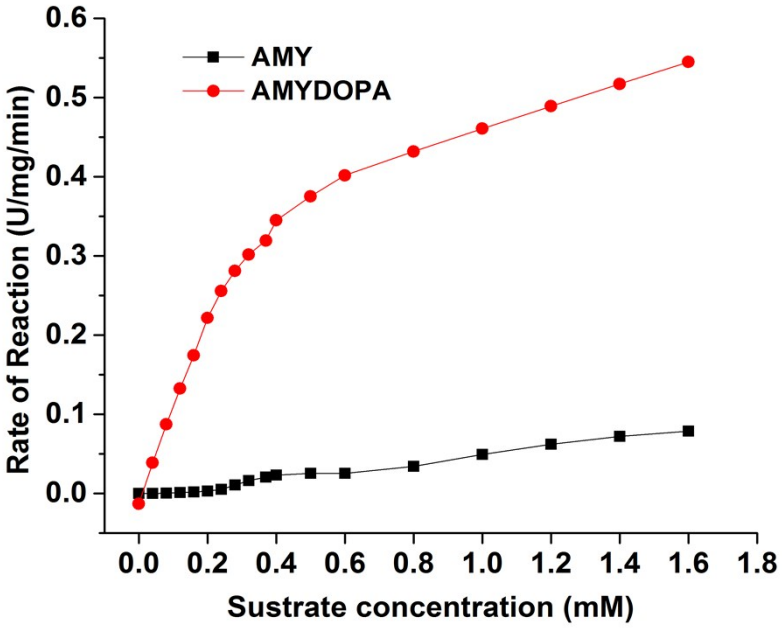


Figure S16: Steady-state enzyme kinetics study was performed and was fitted in Michaelis Menten nonlinear plot to calculate $V_{max,}$ $K_{M,}$ and k_{cat}.

Protein quantification using Lowry's method

1 ml of released content was added to 5 ml of Lowry's reagent (ratio of Na_2CO_3 in 0.1 N NaOH, sodium potassium tartarate, $CuSO_4$ in 100:1:1 respectively) followed by the incubation for 10 min at room temperature. 0.5 ml of 50 % Folin's reagent was added to the mixture and incubated in the dark for 30 min, and absorbance intensity was measured at 640 nm, compared with known BSA standard.

Glycosamine glycans estimation

Dimethyl methylene blue solution (DMMB) was prepared in 1 L water containing 1.6 mg of dimethyl methylene blue, 3.04 g glycine, 2.37 g NaCl and 95 mL of 0.1 M HCl at pH 3. 100 µL of sample was added in 2.5 ml of prepared DMMB solution. Absorbance intensity was taken at 525 nm and compared with standards. Dimethyl methylene blue is a thiazine chromotrope agent that binds to a sulfated GAG, which leads to change in the absorption spectrum due to the induction of metachromasia.

Proteoglycans estimation

1 mL of the sample collected from the recipient cabinet was added to 100 µL of periodic acid solution (50 % of Periodic acid, 7 % of acetic acid) and incubated for 2 hrs at 37 ^{o}C. 100 µL of Schiff's reagent was added with the previously incubated mixer and was kept in the room temperature for 30 min. The intensity was measured at 555 nm and was compared with standards.

Carbohydrate estimation

100 µL of the sample was made up to 1 mL with distilled water. 1 mL of phenol and 5 mL of sulphuric acid were added subsequently. This mixture was incubated for 10 min in shaking condition followed by the incubation at 25-30 °C for 20 min and intensity was measured at 490 nm.

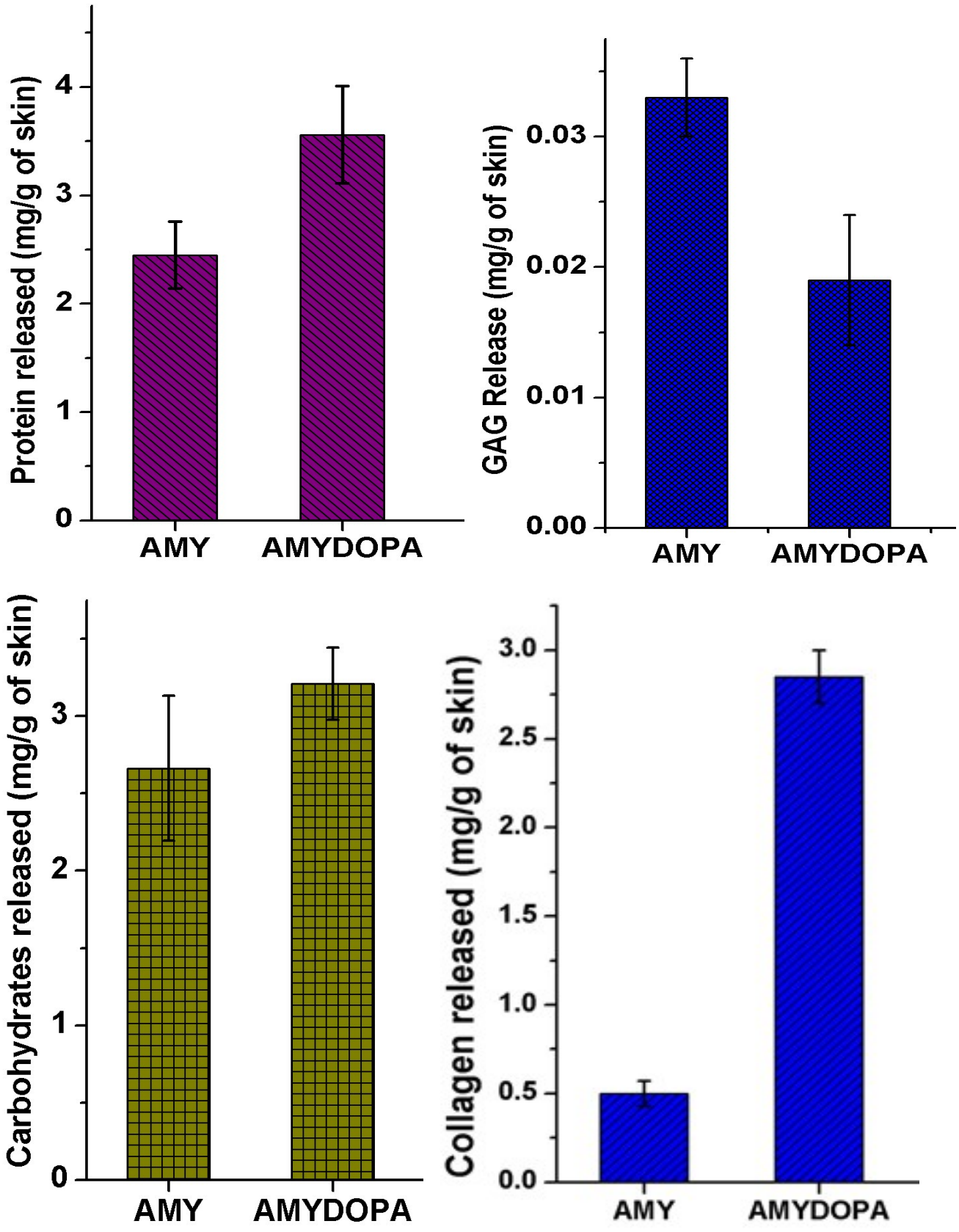


Figure S17: Recipient compartment solutions were collected after enzyme treatment. Protein (A), GAG (B), Carbohydrates (C) and collagen (D) released from enzyme-treated skin were analyzed by standard quantification assay.

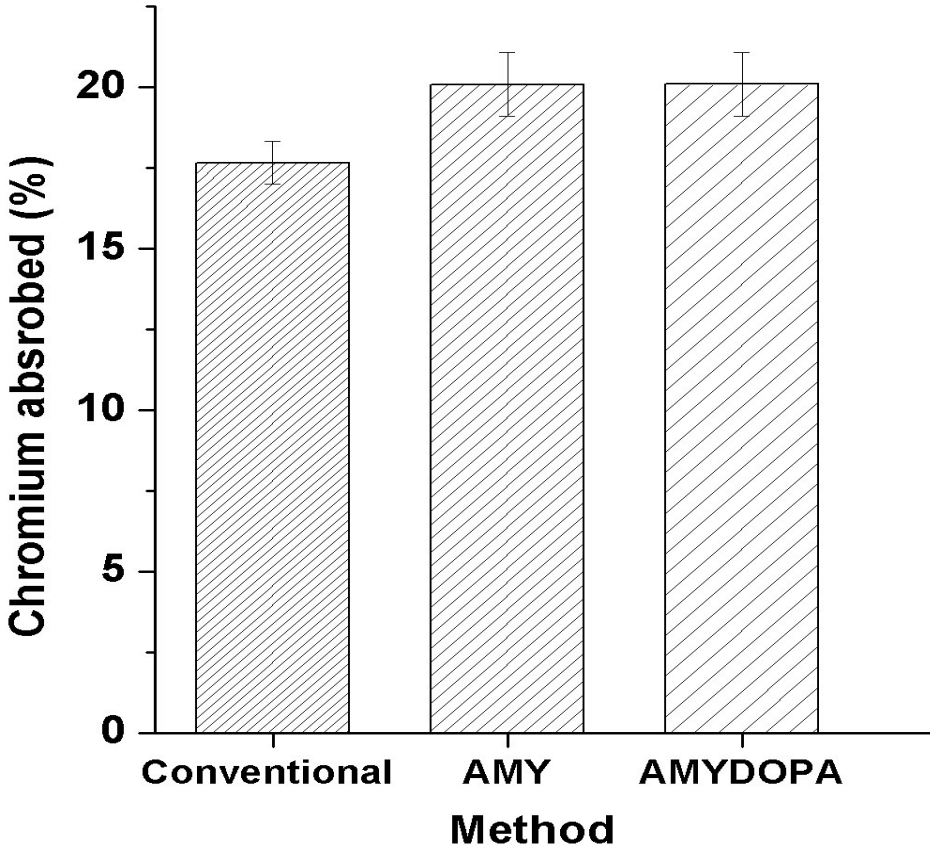


Figure S18: Percentage of chromium absorbed during the tanning process after the various method of pre-tanning process.

Confocal Raman Spectroscopy:

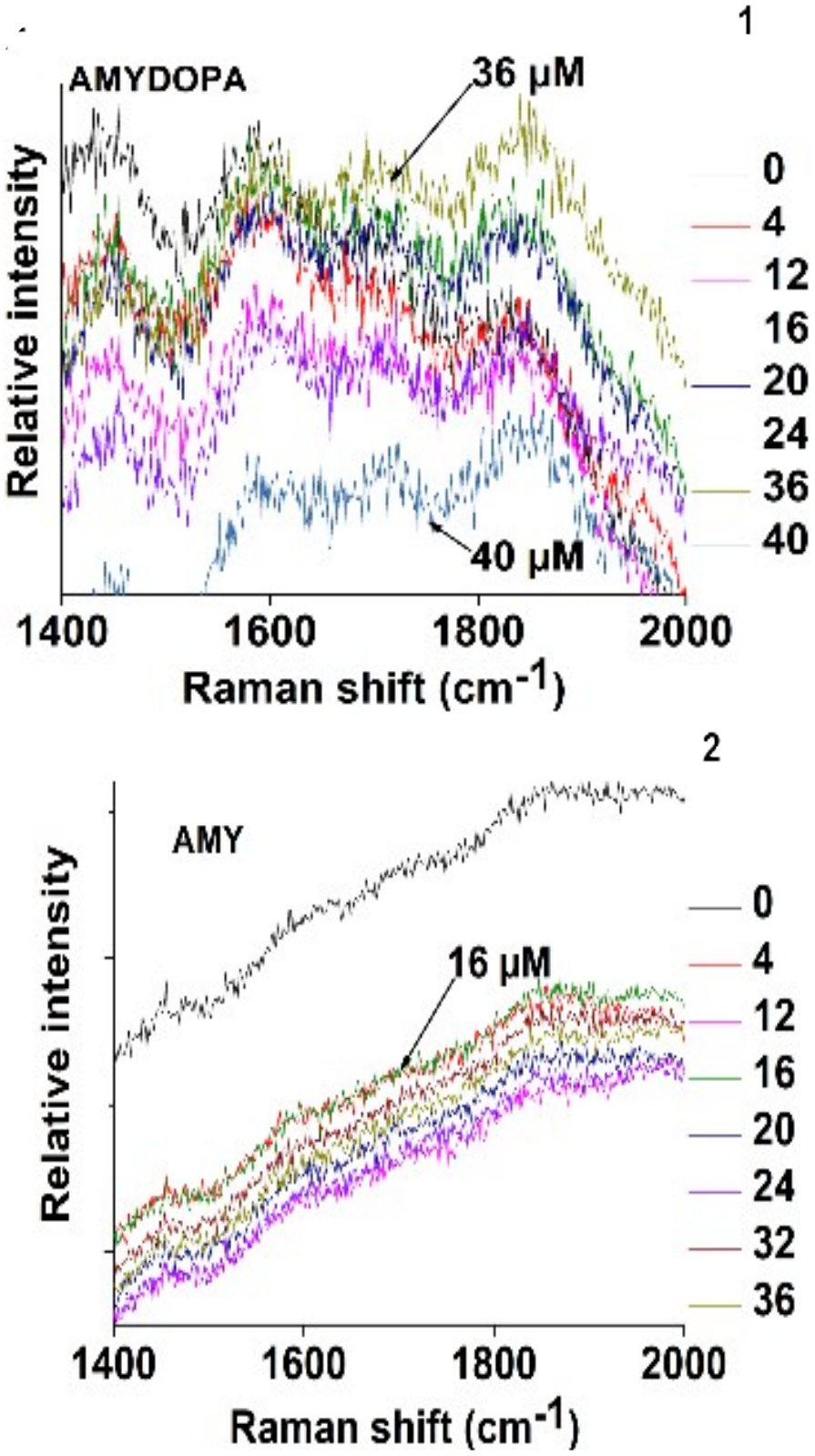


Figure S19: Enzyme AMYDOPA (1) and AMY (2) penetration depth in the skin was analyzed by Confocal Raman Spectroscopy**.**