Electronic Supplementary Information

for

HAP-01, the first chromogenic substrate for *Aspergillus oryzae* acid protease

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Methods for enzymatic assays

I-i. Primary screening of substrate specificity using FRETS-25 substrates

10 μL of koji extract was added to 190 μL of 100 μM FRETS substrate solution (pH 3 "McIlvaine" citric acid-phosphoric acid buffer), and the increase in fluorescence (excitation wavelength 340 nm; fluorescent wavelength 440 nm) in the first 10 min was monitored using a plate leader (Infinite M200 PRO, Tecan Group Ltd., Switzerland) set at 40 °C.

I-ii. Secondary screening of substrate specificity using selected FRETS-25 substrates

10 μL of koji extract was added to 190 μL of 100 μM FRETS substrate solution (pH 3 "McIlvaine" citric acid-phosphoric acid buffer) and the mixture was reacted for 10 min using a plate leader set at 40 °C. Then, 200 μL of 0.4M trichloroacetic acid aqueous solution was added to stop the enzymatic reaction, and the reaction was monitored by LC-fluorescence-MS to estimate the structure and relative amounts of fluorescent enzymatic digestion fragments.

II. Release of pNP from substrates and enzymatic digestion fragments

Two putative substrates and two predicted enzymatic digestion fragments were dissolved in water at 1 mg/50 μL, diluted 100 times with various buffers or water, and left to stand at room temperature, respectively. Reactions were monitored with reversed-phase HPLC after adding 10 μL of trifluoroacetic acid for each 70 μL of solution to stop the reaction.

III. Confirmation of substrate cleavage by koji

HAP-01 was added to koji extract prepared from 70% polished rice and *Aspergillus oryzae* until it reached 100 μM, and the mixture was leaved at 30 °C. At various time points, the aliquot taken from the reaction mixture was analyzed by RP-HPLC after addition of 0.4 M aqueous trichloroacetic acid of the same quantity as the sampled solution to stop the enzymatic reaction.

IV. Quantitative analysis of AP activity using a plate reader.

Enzyme solutions at pH 3 of various *A. oryzae* AP activity (100–600 U/mL) were prepared using many types of koji (various AP activity) according to the standard method established by National Tax Administration Agency (for details, please refer to the following web site (https://www.nta.go.jp/law/tsutatsu/kobetsu/sonota/070622/pdf/211.pdf)), and HAP-01 was added to 500 μM for each solution. After leaved for 20 min at 40 °C, and a sodium carbonate aqueous solution was added and leaved for a further 30 min at 55 °C at pH 10. Then, absorption at 400 nm was monitored.

Characterisation data for HAP-01

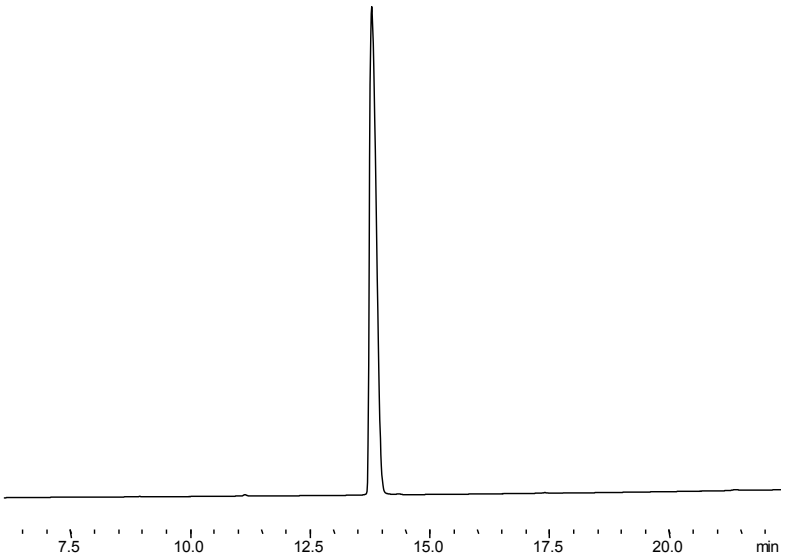

Fig S1. HPLC profile of HAP-01. (Conditions: 1–60% CH_3CN for 25 min (detection at 220 nm) using a Shimadzu liquid chromatograph Model LC-10A with YMC-Pack ODS-A (4.6 x 150 mm) column and the following solvent systems: 0.1% TFA in H_2O and 0.1% TFA in CH_3CN at a flow rate of 1 mL min^{-1} (40 °C))

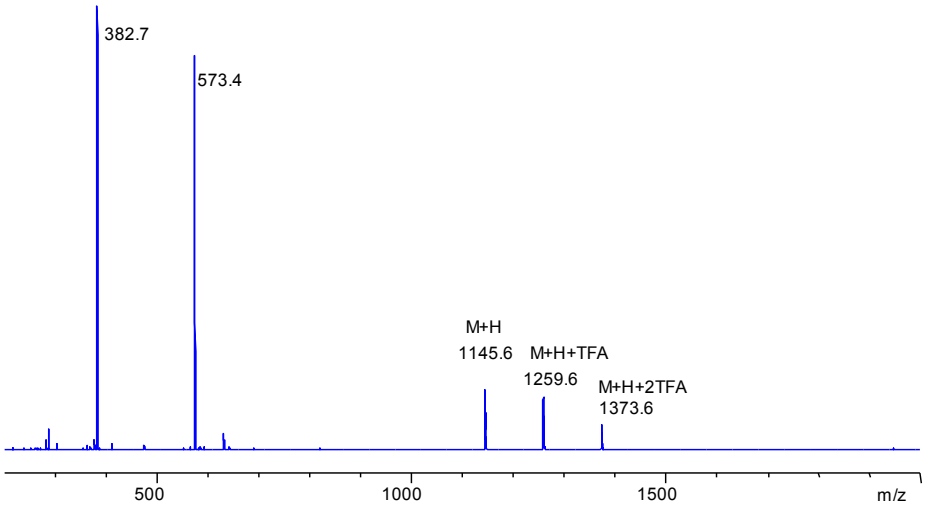

Fig S2. ESI-MS spectrum of HAP-01. (Conditions: an Agilent G1956B LC/MSD detector with an Agilent 1100 series HPLC system)