Structural Determinants of Mucins in Influenza Virus Inhibition: The Role of Sialylated Glycans and Molecular Size

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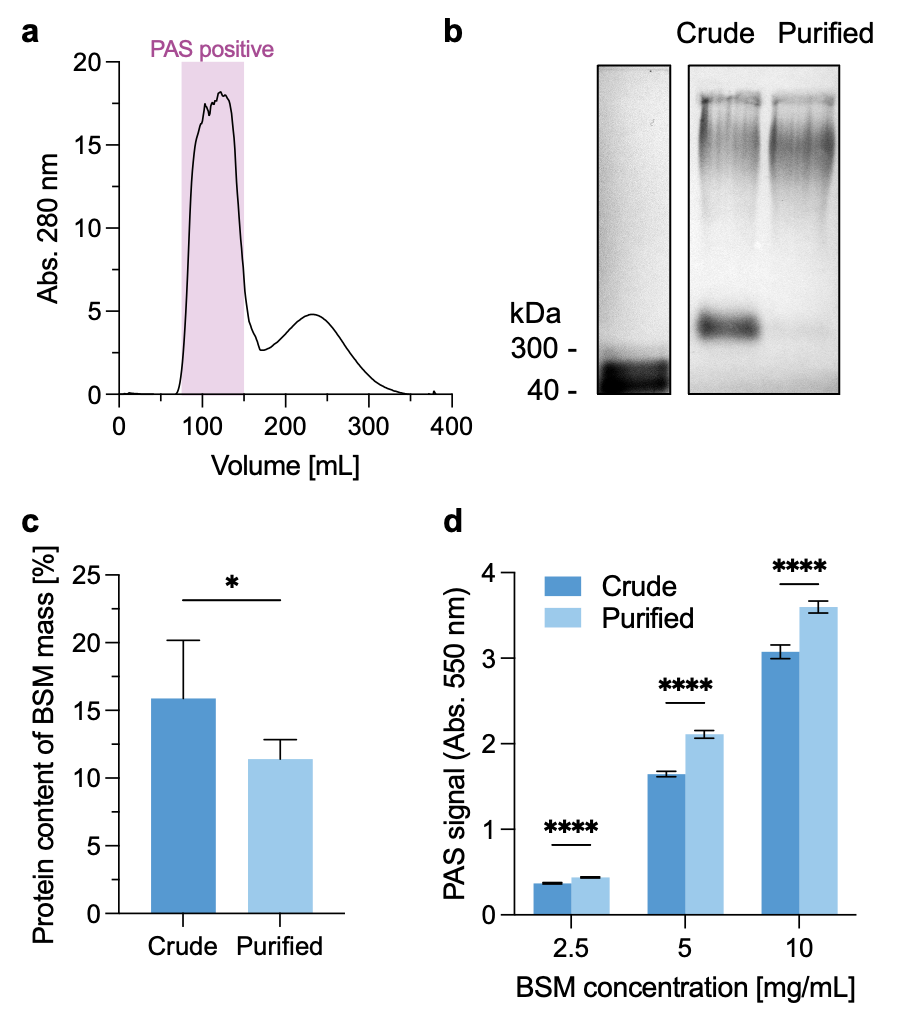
Supporting Information

Figures

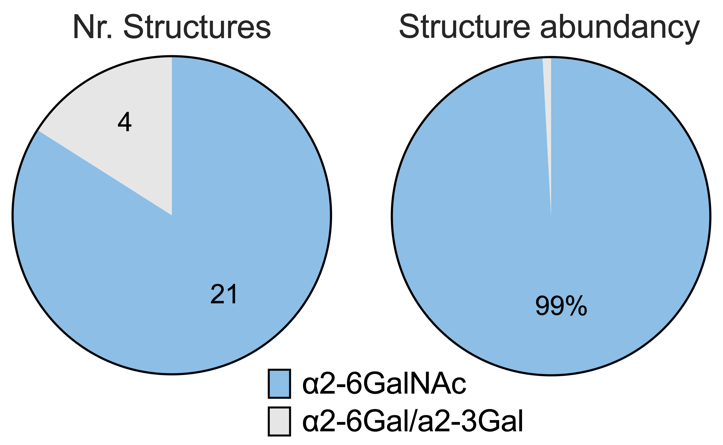
* **Figure S1**: Purification of mucins from bovine submaxillary gland (BSM) by size exclusion chromatography and comparison with crude BSM.
* **Figure S2**: Linkage of sialic acid units in *O*-glycans.
* **Figure S3**: SDS-PAGE of BSM before and after oxidative *β*-elimination.
* **Figure S4**: Comparison of the binding of WGA, SNA, and MAL-II to BSM in a microtiter plate binding assay.
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* **Figure S7**: Sialic acid abundance and density in mucin from bovine submaxillary mucin (BSM) and in mucin fragments.
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* **Figure S9**: Correlation plot between virus inhibition and virus binding.

**Tables**

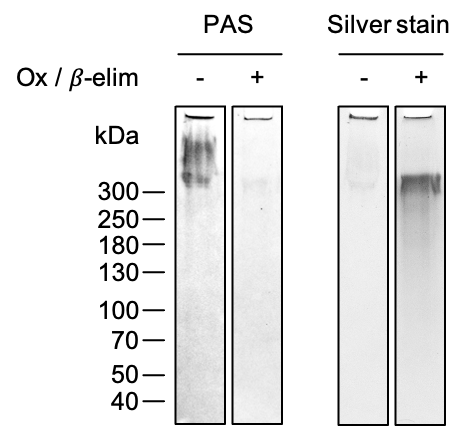
* **Table S1**. Specificity and origin of the proteases used to cleave BSM.



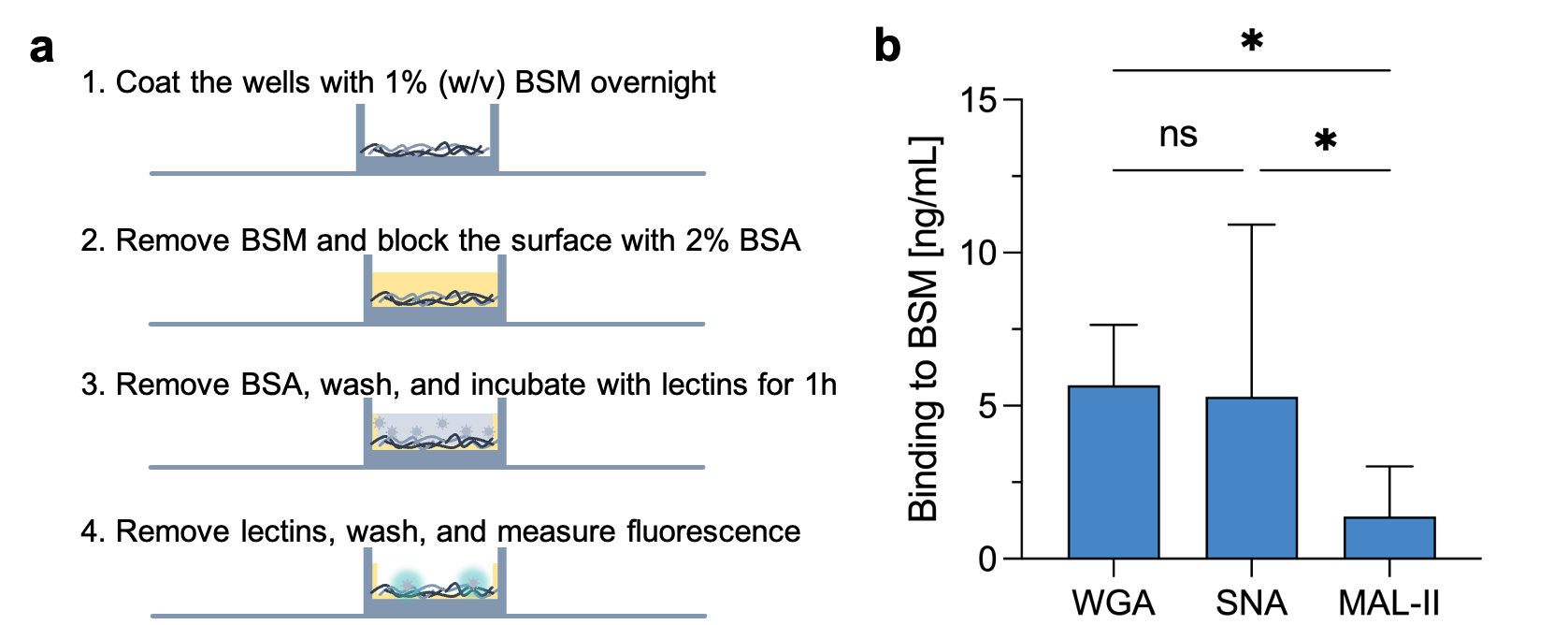
**Figure S1**. Purification of mucins from bovine submaxillary gland (BSM) by size exclusion chromatography and comparison with the crude BSM. (**a**) Size exclusion chromatogram of BSM. Fractions containing mucin were identified by periodic acid/Schiff (PAS) and further enriched. (**b**) Agarose gel electrophoresis of BSM before and after purification. Samples are stained with PAS. Equal masses (30 µg) of samples were loaded. The lane of the protein marker is cropped from the same blot. (**c**) Protein content of a 10 mg/mL BSM sample measured with the bicinchoninic acid assay (BCA). Student’s t-test is used to compare the means between two groups. (**d**) PAS signal measured as absorbance at 550 nm of the BSM samples before and after purification, at different concentrations of dry mass of BSM. Multiple t-test was used to compare the means of the crude and purified groups. Results are presented as mean values ± standard deviation (SD) of N = 3. *p* < 0.05 (\*), *p* < 0.01 (\*\*), *p* < 0.001 (\*\*\*), *p* < 0.0001 (\*\*\*\*).



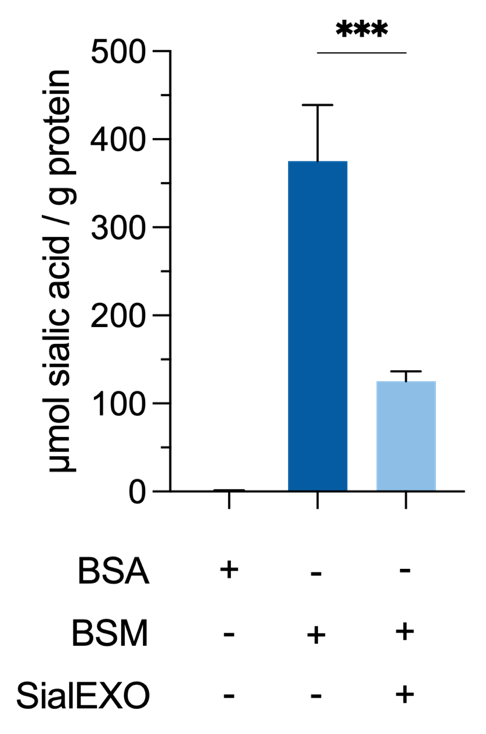
**Figure S2.** Distribution of the sialic acid linkages among the resolved *O*-glycans structures.Frequency of SAα2-6GalNAc is depicted as total number of *O*-glycans with SAα2-6GalNAc, and as total abundancy of SAα2-6GalNAc based on the individual abundance of the *O*-sialoglycan. For four low-abundant *O*-glycans (<1%), accurate assignment of the SAα2-3Gal or α2-6Gal linkage was not possible due to limitations in the MS/MS fragmentation patterns.



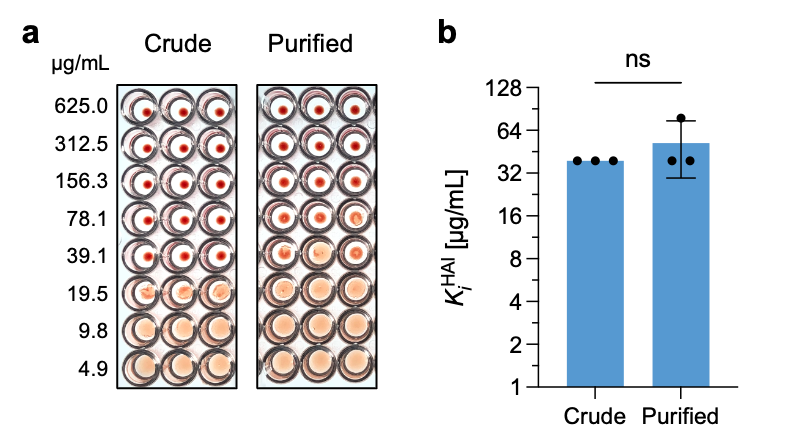
**Figure S3**. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of purified mucin from bovine submaxillary gland before (-) and after (+) oxidative *β*-elimination for glycan removal. Gels are stained with periodic acid-Schiff (PAS) or silver staining to highlight glycans and protein, respectively. A total of 5 µg of BSM was uploaded in each lane.



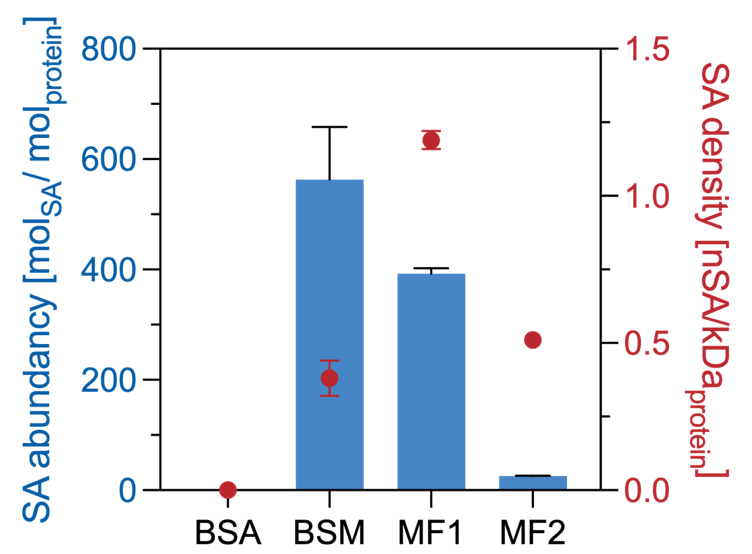
**Figure S4**. Comparison of the binding of fluorescently labelled wheat germ agglutinin (WGA), Sambucus nigra lectin (SNA), and Maakia amurensis lectin (MAL-II) to BSM in a microtiter plate binding assay. (**a**) Schematic representation of the processes of lectin binding to BSM. At first, 100 µL of 1% (w/v) of BSM in DPBS is inserted into the wells and incubated overnight at 4 °C. Next, the BSM sample is removed and the wells are washed twice with washing buffer (DPBS containing 0.1% w/v twice bovine serum albumin, BSA) followed by addition of 100 µL of blocking solution (2% (w/v) BSA in DPBS) to block the surfaces for 1 h at room temperature. The blocking solution is discarded, the wells are washed twice with washing buffer, followed by incubation for 1h with 100 µL of 1 µg/mL fluorescently-labelled lectin solution. The lectin solution is then discarded, the wells are washed twice with washing buffer, and the fluorescence is measured at lectin’s specific wavelength. The amount of lectin bound is calculated using a lectin-specific calibration curve. (**b**) Amount of lectin quantified in the microtiter plate binding assay. The bars represent the average (± SD) of N=12 wells. Statistical significance is calculated using one-way ANOVA, *p* < 0.05 (\*), *p* < 0.01 (\*\*), *p* < 0.001 (\*\*\*), *p* < 0.0001 (\*\*\*\*).



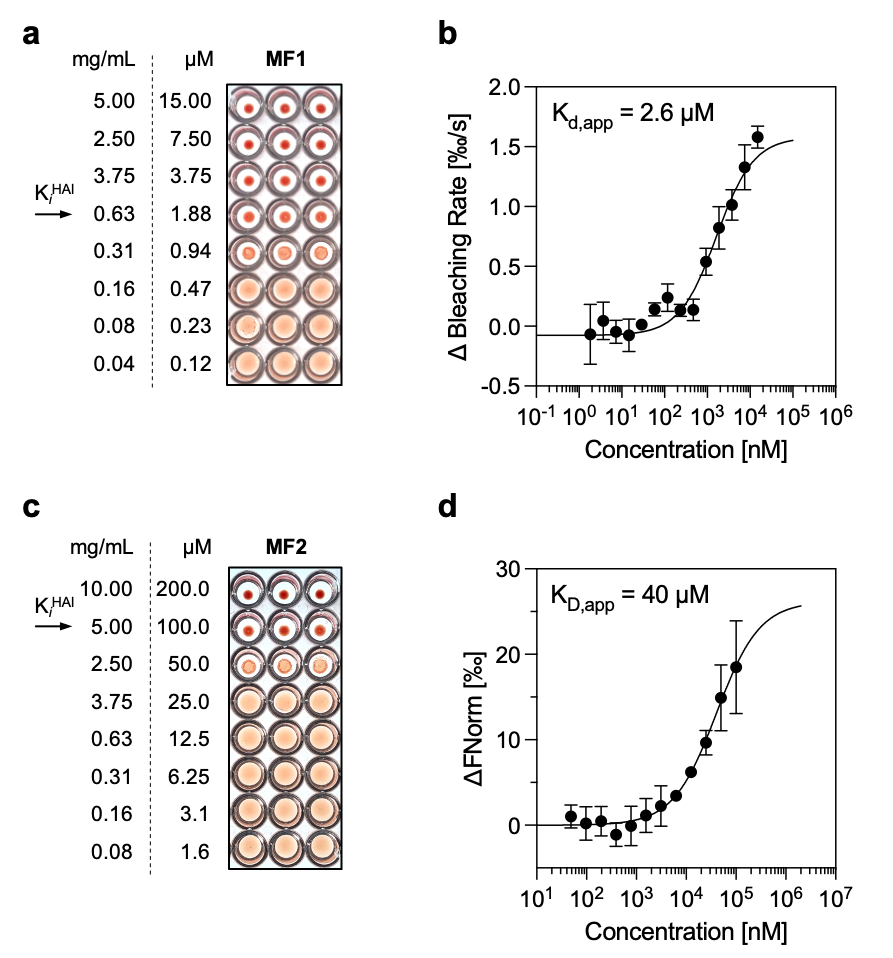
**Figure S5**. Sialic acid quantification after sialidase (SialEXO®) treatment of BSM. Bovine serum albumin (BSA) was used as control of non-glycosylated protein (negative control). Results are reported as mean of N=3 independent measurements ± standard deviation (SD). The significance level was calculated using the Student’s t-test. *p* < 0.05 (\*), *p* < 0.01 (\*\*), *p* < 0.001 (\*\*\*), *p* < 0.0001 (\*\*\*\*).

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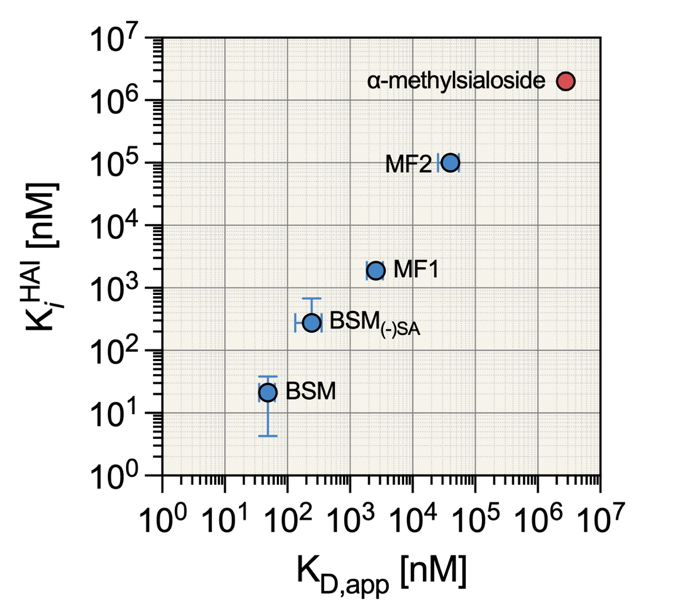
**Figure S6**. Comparison of the inhibition activity of crude and purified BSM. (**a**) Hemagglutination inhibition assay using A/Panama/2007/1999(H3N2) virus. (**b**) Bar plot of the lowest log2 inhibitor concentration necessary to achieve complete inhibition of hemagglutination caused by the virus.



**Figure S7**. Sialic acid abundance (mol of sialic acid per mol of protein) and density (number of sialic acid residues per kDa of protein) in mucin from purified bovine submaxillary mucin (BSM) and in the mucin fragments obtained by StcE (MF1) and proteinase-K (MF2) digestion. Sialic acid abundance was measured using the NANA assay and quantified using a sialic acid five-points calibration curve (R2=0.99). Conversion of mass/volume into molarity concentrations of BSM, MF1, and MF2 was calculated assuming a molecular weight (MW) of 1500, 330, and 50 kDa respectively, according to the apparent MW observed on gel electrophoresis. Bovine serum albumin (BSA) was used as negative control. Results are reported as mean of N=4 independent measurements ± standard deviation (SD).



**Figure S8**. Antiviral activity and binding to four hemagglutination units of influenza A/Panama/2007/99 (H3N2) of mucin fragments obtained by digestion with StcE (MF1) and proteinase-K (MF2). Hemagglutination inhibition assay (N=3), and binding curve obtained from microscale thermophoresis analysis of octadecyl rhodamine B chloride (R18) labelled X/31 virus (4 HAU) with MF1 (**a, b**) and MF2 (**c, d)**. Data points were fitted according to the mass-action law function to calculate KD,app values.Results reported as mean of N=4 independent measurements ± standard deviation). Conversion of dry mass/volume into molarity concentrations of MF1 and MF2 was calculated assuming a molecular weight (MW) of 330 and 50 kDa respectively, according to the apparent MW observed on gel electrophoresis (see Figure 5b).



**Figure S9**. Correlation plot between virus inhibition and virus binding. Scatter plot depicting on y-axis the inhibition constant (K*i*HAI, mean of N=3 ± SD) measured by hemagglutination inhibition assay, and on x-axis the apparent dissociation constant (KD,app, mean of N=4 ± SEM) determined from microscale thermophoresis measurements. BSM = mucin from bovine submaxillary gland; BSM(-)SA = BSM after treatment with sialidase to remove sialic acid; MF1 = mucin fragment obtained from StcE digestion; MF2 = mucin fragment obtained from proteinase-k digestion. α-methylsialoside (red dot) is reported from [1] and is used as ligand with minimum affinity and activity in the assay.

**Table S1.** Specificity and origin of the proteases used to cleave BSM.

|  |  |  |  |
| --- | --- | --- | --- |
| **Protease** | **Family** | **Specificity** | **Reference** |
| SmE | Metalloprotease | N-terminally to a glycosylated Ser or Thr residue. (Different O-glycans at the P1’ position are accepted). | [2] |
| StcE | Metalloprotease | N-terminally to a Ser/Thr\*-X-Ser/Thr, where the cleavage happens on the amino side of the 2nd Ser/Thr, and X can be any amino acid, or none. | [3] |
| Pepsin | Aspartic protease | Broad specificity: preference for cleavage C-terminal to Phe, Leu, and Glu. | [4] |
| Chymotrypsin | Serine protease | C-terminal of hydrophobic residues (*e.g*., Phe, Tyr, Trp, Leu). | [4] |
| Trypsin | Serine protease | C-terminal of Lys and Arg residues, except for -Arg-Pro- and -Lys-Pro- bonds which are normally resistant to proteolysis. | [4], [5] |
| Elastase | Serine protease | C-terminal of amino acids with small hydrophobic side chains (e.g., Ala, Val, Ser, Gly, Leu, Ile) | [4] |
| Proteinase K | Serine protease | Broad specificity: C-terminal of aliphatic and aromatic amino acids. | [4,6] |
| Bromelain | Cysteine endopeptidase | Broad specificity: cleaves at the C-terminal, high efficiency on Arg-Arg-containing substrates. | [7] |
| Papain | Cysteine endopeptidase | Broad specificity: cleaves at the C-terminal, preference for amino acids bearing a large hydrophobic side chain at the P2 position. Does not accept Val in P1'. | [8] |
| Ficin (ficain) | Cysteine endopeptidase | Broad specificity: cleaves at the C-terminal, accepts Gly, Ser, Thr, Met, Lys, Arg, Tyr, Ala, Asn, and Val, with preferences for hydrophobic side chains. | [9] |

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