Biophenol–Protein Supramolecular Models by Fast Atom Bombardment-Mass Spectrometric Experiments

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Biomimetic supramolecular formation between hydroxytyrosol, a biophenol found in olives and virgin olive oil, and caffeine or Asp-Phe, as proteic models, has been achieved by FAB-mass spectrometric experiments. The protonated supermolecules show a consistently higher difference in stability constants, thus indicating a preferential molecular recognition site provided by caffeine, the biomimetic model of proline-rich mucoproteins. The spontaneous aggregation of the complementary supramolecular components suggests correlations with the sensorial response and the bioavailability of food biophenols.

Keywords: *Biophenols; biomimetic molecular recognition; supermolecules; mass spectrometry*

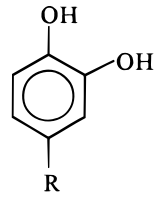
INTRODUCTION

Molecular microcomponents of Mediterranean foods (Mf) show renewed interest, since they exert many physiological effects in human nutrition and affect the quality and authenticity of traditional products (Angerosa et al., 1995). In the vegetable kingdom, biophenols are widely distributed and are present in relative amounts in fruits, leaves, and other vegetable organs. Their molecular activity, as tastant, antioxidative, and chelating functionality, enhances the defensive and protective factors, preserving plant tissues from herbivorous attack (Baxter et al., 1996).

Biophenolic ingredients are naturally found in olives and virgin olive oil and are lost in other refined and seed oils during the various processing stages (Montedoro and Cantarelli, 1969). This non-nutritive portion of the typical Mediterranean diet assumes a fundamental role for the product quality and for its relation to oxidation resistance and the peculiar bitter taste, with further beneficial health effects (Casuscelli et al., 1994). The functional groups of **1** affect sensory and nutritional features of virgin and processed oils (Olias, 1992), being a tastant substrate (St) in their sensorial perception, with a variety of stimuli (bitter, pungent, spicy, sour, astringent) in agreement with its structure and molecular complexity (Shallenberger, 1993).

Biophenols **1**, before eliciting their biological activity, need to reach the proper target in the human body, through a long and complicated journey: beginning from the olive plant, via the mouth, and then down to the cells. Oleuropein, the most important biophenol in olive fruit, and its metabolite demethyloleuropein (Gariboldi et al., 1986) release the 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol, Ht) moiety by enzymatic hydrolysis (Limiroli et al., 1995). In the mouth, Ht may bind to sensorial receptors (Rs) on the tongue and to mucoproteins (Pm) and other food ingredients (If) in the oral cavity (van der Heijden, 1993). These interactions form supermolecules with globular and proline-rich Pm. The specific supramolecular interaction among Ht, Rs, Pm, and other If may involve absorption and desorption equilibria with formation of charge transfer host/guest aggregates.

Chart 1. General Formula of Biophenols



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Molecular biomimetic experiments with olive biophenols have been undertaken in order to rationalize the bioactivity of these microingredients and to predict their destination, bioavailability, and sensorial interaction. The biomimetic models to be experimented should have specific features, providing the Ht substrate of appropriate sites to be linked with electrostatic forces, hydrogen bonds, and/or noncovalent interactions, and should have hydrophobic location for biological flexibility and specificity.

The interaction of Ht with Rs, Pm, and some If has been studied by the biomimetic model Ht interacting with caffeine (C) or the dipeptide Asp-Phe (Ap), as Rs, Pm, and some If possess proteic structure. Caffeine has been selected for its easy association with monomeric and polymeric biophenols, competing effectively with proteins in the process of supramolecular formation (Baxter et al., 1996). In fact, C is characterized by a

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molecular structure, reminiscent of proline-rich Pm, in its −CO−N(Me)− structural unit, due to the carbonyl and the tertiary peptide-like nitrogen and its availability for hydrophobic sites. Ap models the different conformational flexibility, shown by globular Rs and If proteic structures, with respect to proline-rich Pm.

The biomimetic phenomenon has been evaluated using fast atom bombardment (FAB)-mass spectrometry by monitoring the supermolecule formation through the abundance of the corresponding protonated molecular ions: $[Ht-C-H]^+$ and $[Ht-Ap-H]^+$.

MATERIALS AND METHODS

Hydroxytyrosol Extraction. *From Olives.* Biophenolic compounds from olives have been obtained by subsequent extractions with methanol, methanol/water (60:40), hexane, chloroform, and ethyl acetate. Five hundred grams of crushed olives was extracted three times with 100 mL of methanol by stirring on a magnetic plate for 15 min. After filtration, the solvent was removed under reduced pressure at $T < 60$ °C and the residue dissolved in 100 mL of methanol/water (60:40, v/v). The hydroalcoholic extract was washed with hexane (3 × 50 mL) and chloroform (3 × 50 mL). The solvent was removed under reduced pressure ($T < 60$ °C); the residue was taken up with ethyl acetate and separated on a silica gel column using as eluent a mixture of $CHCl_3$/MeOH gradually varied from 99:1 to 90:10 every 200 mL. The obtained amount of hydroxytyrosol was 5.8 mg/kg.

From Olive Oil. Three hundred grams of olive oil was dissolved in 300 mL of hexane and extracted three times with 100 mL of a methanol/water (60:40, v/v) solution. The lower layers were combined and evaporated under reduced pressure at $T < 60$ °C. The residue was separated on a silica gel column using a mixture of 95:5 $CHCl_3$/MeOH to give 1.92 mg of pure hydroxytyrosol.

Hydroxytyrosol Synthesis. Hydroxytyrosol was synthesized from 3,4-dihydroxyphenylacetic acid (Fluka Chemie AG, Buchs, Switzerland) in two steps: acetylation of the phenolic function and subsequent $LiAlH_4$ reduction. Crude hydroxytyrosol was then chromatographed on a silica gel column using MeOH/$CHCl_3$ (1:9) as an eluent to give the pure hydroxytyrosol in 70% yield.

Mass Spectrometry. All the spectra were obtained with a VG-ZAB-T four-sector instrument equipped with a FAB source. Cesium ion gun was used with 30 kV of accelerating voltage. The ions were accelerated with 8 kV of potential. In the MS1 experiments the ion beam was recorded after the first two sectors (B1E1) on a normal single-point photomultiplier detector. For data processing the OPUS V3.IX software was used. The samples were dissolved in methanol, and 1 μL of the solution was applied on the probe tip in 1 μL of glycerol matrix. Several scans were acquired and added together. The MS/MS experiments were run with Ar as the collision gas in the collision cell placed after the first electrostatic analyzer. The pressure of the collision cell was set to 75% attenuation of the selected parent peak. The daughter spectrum was analyzed with the magnetic and focal plan electrostatic sectors (B2E2) situated after the collision cell. For high-sensitivity detection an array detector was used. The arrangement between the focal plan ESA2 and the array detector was set to 30°.

RESULTS AND DISCUSSION

Host/guest aggregates are frequently examined by mass spectrometry: these applications have been reviewed recently (Vincenti, 1995). The binding of small molecules or ions to a large one is often observed, like partial solvation of macromolecules. Peptides, proteins, and nucleic acids also form dimers or oligomers: such species can be detected by MS. Formation of supermolecules may involve relatively small molecules, like crown ethers and amines, and was found to depend on such fine structural features as chirality (Pòcsfalvi et al., 1996; Dobò et al., 1997).

Supermolecules can be studied by a variety of mass spectrometric techniques. Most often FAB or electrospray ionization is used, altough electron impact, chemical ionization, and matrix-assisted laser desorption ionization (MALDI) are also applicable (Russell et al., 1995). The detection of a dimer (or oligomer) does not necessarily mean a specific strong binding. Adducts may be formed due to loose and unspecific H-bonds or van der Waals forces as well. The formations of oligomers of matrix molecules and their adducts with the analyte in FAB are well-known examples.

The specificity of binding in a dimer can be checked by a variety of techniques, most often by ion abundance ratios observed in single stage or tandem mass spectra. To obtain meaningful results, comparison of values measured for various compounds or homologues is highly desirable. Instead of relative peak intensities (Sawada et al., 1992), the use of stability constants calculated for ion abundances was found to be advantageous (Winkler et al., 1986; Pòcsfalvi et al., 1996; Dobò et al., 1997). This approach involves the use of ion abundances and the formalism of a chemical equilibrium, but the presence of a true equilibrium is not a prerequisite for its application. As the results suggested (Pòcsfalvi et al., 1996; Dobò et al., 1997), errors mostly cancel and the relative stability constant so derived will characterize the relative stabilities of various molecular aggregates. This relative stability constant should not, however, be regarded as a thermodynamic value.

The FAB spectrum of Ht−C and Ht−Ap mixtures have been studied. Due to the low proton affinity of hydroxytyrosol, its protonated molecular ion ($[Ht + H]^+$, m/z 155) is of low abundance. Protonated dimers of hydroxytyrosol, $[Ht_2 + H]^+$, caffeine, $[C_2 + H]^+$, and Ap, $[Ap_2 + H]^+$, are also observed, just as the mixed dimers of hydroxytyrosol−caffeine, $[Ht-C-H]^+$, and hydroxytyrosol and Ap, $[Ht-Ap-H]^+$. In the present context these dimers will be studied and compared; the relevant parts of the FAB spectra are shown in Figures 1 and 2. All other major peaks in the spectra were identified as protonated molecular ions, fragments, and glycerol-containing complexes, but these will not be discussed here.

The protonated dimers may be formed through various reaction channels, e.g., formation of a neutral dimer followed by protonation or protonation of a monomer followed by association with a neutral species. The stability of a protonated dimer, ABH^+, can be characterized by the stability constant (K_{AB}*) defined in the following way:

$$A + B + H^+ \rightleftharpoons ABH^+$$

$$K_{AB}^* = \frac{[ABH^+]}{[A][B][H^+]} \quad (1)$$

A and B indicate the neutral monomers and square brackets the concentrations (or, more precisely, activities). Similar equations can be written for the formation of homodimers. However, these equations can not directly be used for characterizing the complex stability as the concentration of protons is an abstract value and the concentration of neutrals at the time and place of ion formation can not simply be determined by mass spectrometry. The stability of the heterodimer ABH^+ can, however, be compared to the stability of ho-

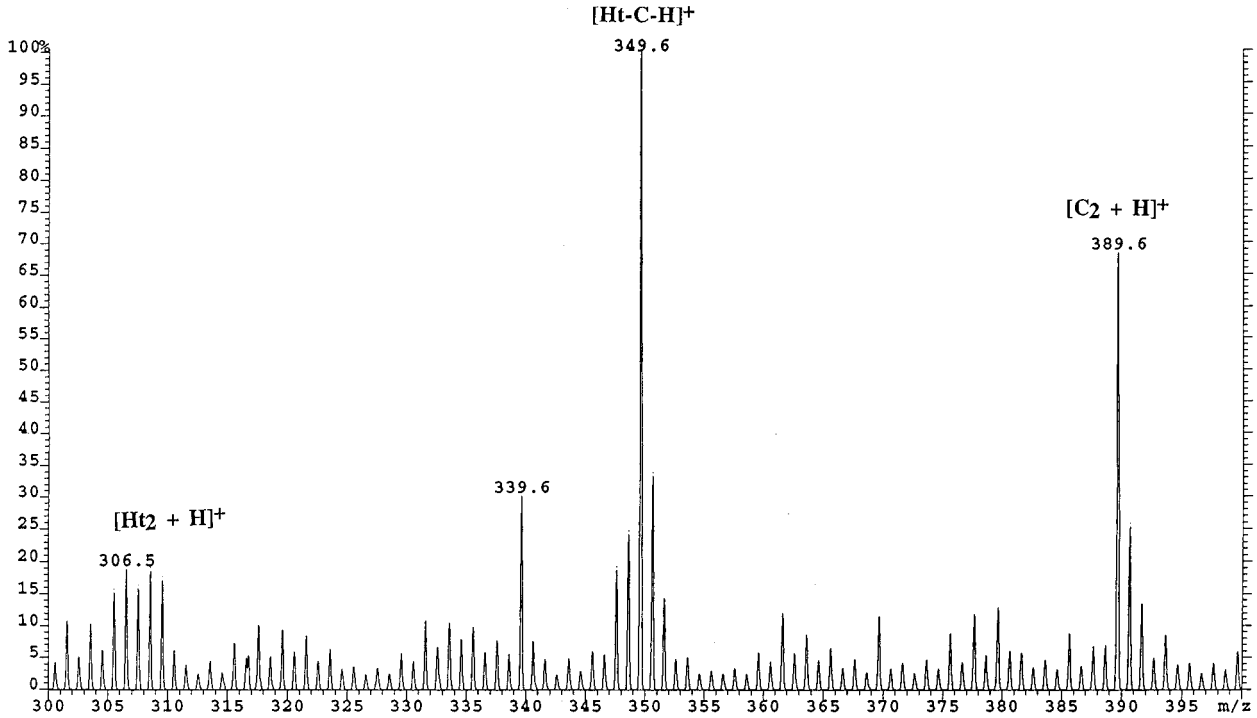

Figure 1. FAB-MS spectrum of caffeine and hydroxytyrosol mixture.

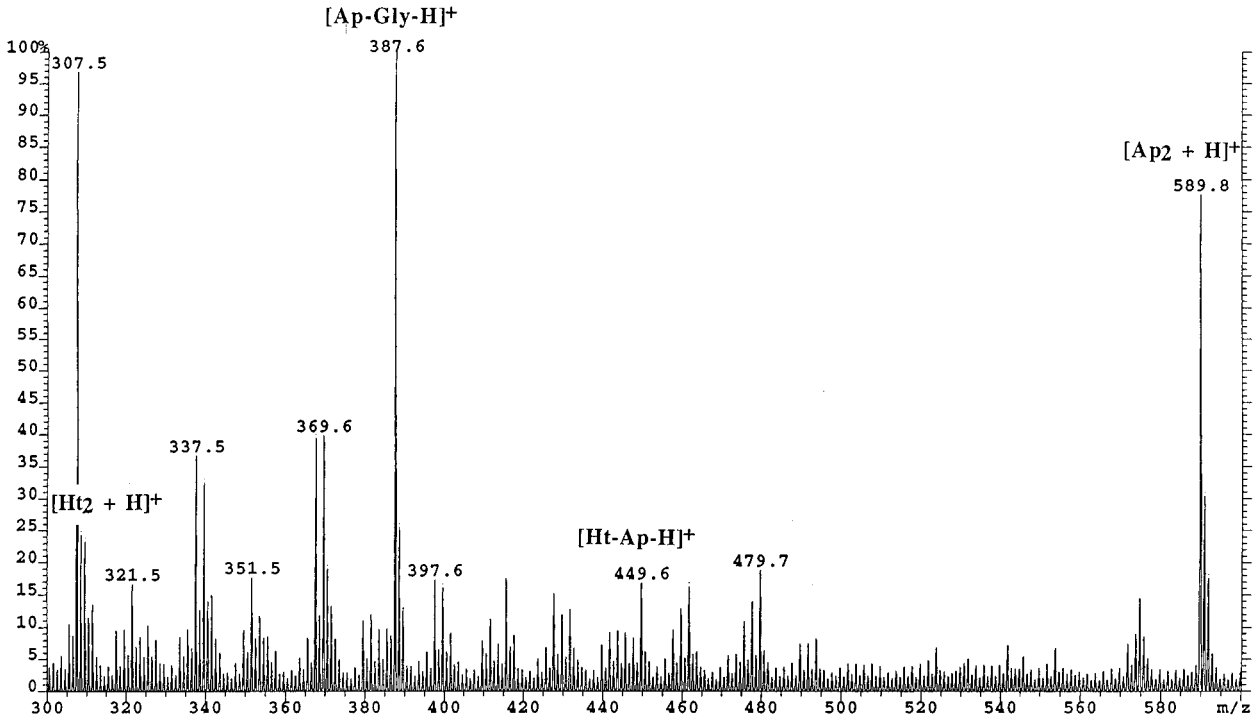

Figure 2. FAB-MS spectrum of Asp-Phe and hydroxytyrosol mixture.

modimers A_2H^+ and B_2H^+ in the following way:

$$K_{AB} = \frac{K_{AB}^*}{2\sqrt{K_{A2}^* K_{B2}^*}} = \frac{[ABH^+]}{2\sqrt{A_2H^+][B_2H^+]}} \qquad (2)$$

K_{AB}, expressed in this way, contains concentration of protonated dimers only. The concentration ratio can be approximated by the abundance ratio of the ions, measured in the spectrum. Although this correlation is not always straightforward, the assumption that peak ratios reflect the relative concentrations is nearly always used in the evaluation of mass spectral data: the more similar are the ions and the experimental conditions, the better this relationship will hold. If the abundance ratios of two protonated dimers are compared, the insertion of ion abundances instead of concentrations in eq 2 is likely to be a good approximation. This approach was applied in cases where very small differences (less than 1 kJ/mol) in the stability between adduct ions have been successfully distinguished (Pòcsfalvi et al., 1996; Dobò et al., 1997). It should be noted that the knowledge on the mechanism of protonated dimer formation is irrelevant and the presence of true equilibrium conditions in ion formation is not necessary for the application of eq 2.

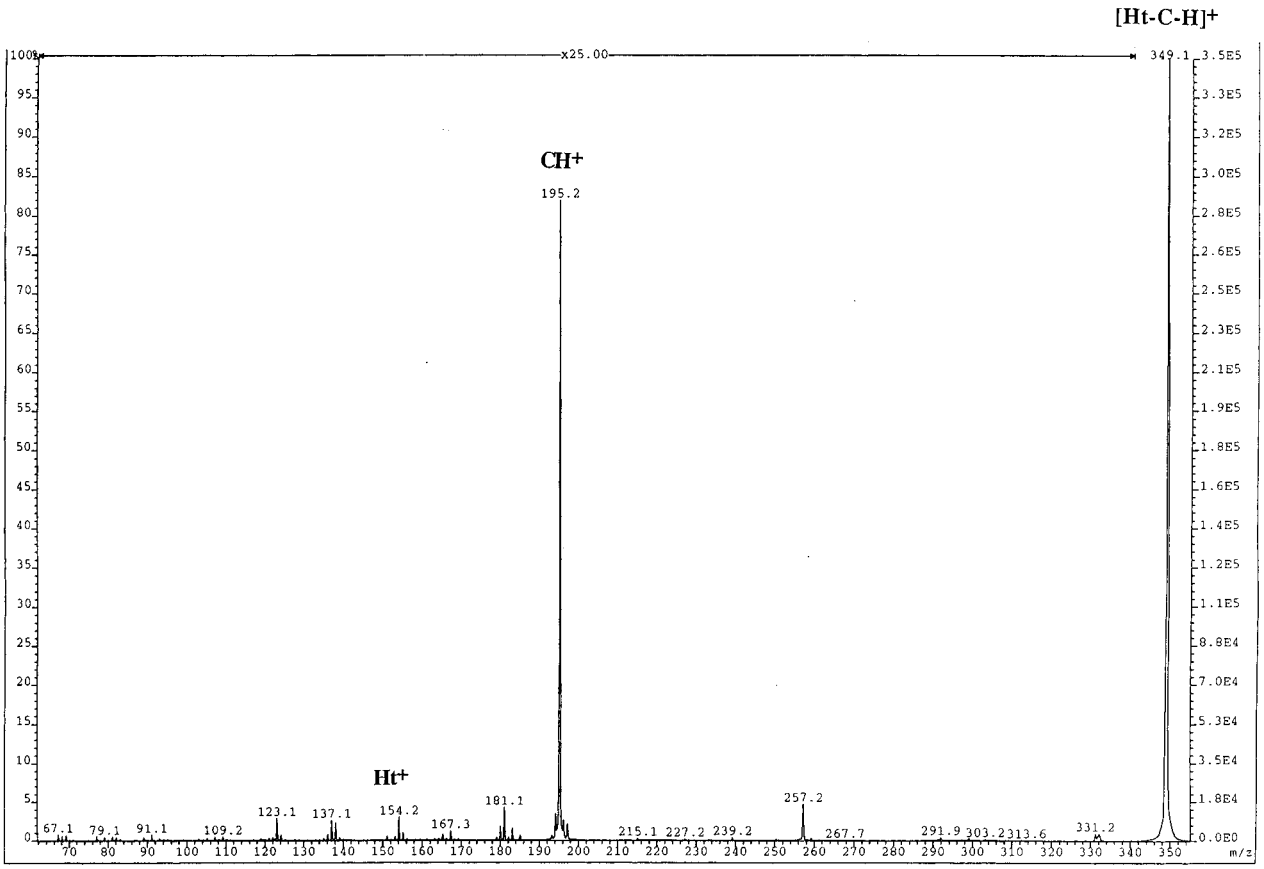

Figure 3. CID MS/MS spectrum of caffeine−hydroxytyrosol supermolecule.

K_{AB} expressed in eq 2 will be equal to unity, if formation of ABH^+, A_2H^+, and B_2H^+ would be statistical (the three dimers would be of equal stability). A K_{AB} value larger than 1 indicates favorable bonding and a specific structure of the heterodimer. $K_{AB} < 1$ indicates, on the other hand, that the heterodimer is less stable than the homodimers. Similar arguments and equations have been developed and used in various other studies on host/guest chemistry by MS. A famous example is the first study on enantiomers by MS; other cases have also been discussed before (Fales and Wright, 1977).

In the case of the hydroxytyrosol−caffeine mixture (Figure 1), the relative stability constant K_{Ht-C} is 1.7, a value much higher than unity. This indicates that hydroxytyrosol forms a particularly strong supermolecule with caffeine, suggesting special molecular recognition between these two species.

To test the validity of the present approach to characterize supramolecule formation, the mixture of Ht and Ap was also studied (Figure 2), in order to evaluate the molecular recognition attitude of Ht toward the systems C and Ap, as biomimetic models of the different proteins present in the oral cavity. In the case of Ap, the heterodimer has a lower abundance than that expected statistically based on the homodimers. The corresponding relative stability constant (K_{Ht-Ap}) is small, 0.16, indicating that the $Ht-Ap-H^+$ supermolecule is bound together only by weak and nonspecific interactions. A similar case was found also in the mixture of C and Ap: the relative stability constant K_{C-Ap} was 0.3, indicating that no specific supermolecule was formed.

Changing the concentration of the interacting compounds modifies peak abundances significantly. Increasing the concentration, the intensities of adduct peaks increase, compared to that of the monomers, as can be expected. The relative stability index, determined according to eq 2, varies only slightly with concentration. One order of magnitude change in the concentration influences the stability index (K_{Ht-C}) by less than 30%.

The unimolecular chemistry of the protonated heterodimer $Ht-C-H^+$ was studied by tandem mass spectrometry as well (Figure 3). The largest peak in the CID MS/MS spectrum of the protonated heterodimer is the protonated molecular ion of caffeine ($C-H^+$). This can be explained either by the higher proton affinity of caffeine than that of hydroxytyrosol or by dimer formation followed by protonation at the caffeine portion or a combination of these effects.

CONCLUSIONS

A large number of natural derivatives of **1**, found in Mf, is provided in the meals, some of them derived from olives and virgin olive oil. Mastication breaks down Mf into small particles, increasing the surface area and releasing biomolecular components from Mf. Concomitant saliva secretion dilutes molecular Mf ingredients, transports them within and out of the oral cavity, and facilitates interaction with the proteins of sensorial receptors on the tongue. Proteins of saliva secretion are characterized by an open-flexible structure with a corresponding great potential for association, because of the high levels of proline.

The molecular experiments with biomimetic C and Ap components of supermolecules with biophenols **1** relate to the application of molecular models for understanding biological food processing, i.e., the interaction of the St

with the tongue papillae and its association with If and proline-rich Pm. The rationalization of the biomimetic interactions, shown by FAB-MS and quantified by the relative stability constants, observed under FAB-MS conditions indicates spontaneous supramolecular formation between olive biophenol Ht and caffeine, the biomimetic model of proline-rich mucoproteins. The molecular affinity of these two supramolecular components was shown to be consistently greater than that of Ht with Ap, the biomimetic model of globular and low-flexible proteins.

The FAB-MS experiment thus performed gives a suggestion for the behavior of biophenols toward the proteins present in the oral cavity. At the physiological pH (6.2−7.4) in the mouth, the protonated supermolecule $Ht-CH^+$ is highly stabilized compared to the $Ht-ApH^+$ one; the olive biophenol Ht is likely to reside at the open-flexible moiety of the proline-rich Pm, having a good site for supramolecular interaction. The differential partitioning of Ht among the proteic components Rs, If, and Pm may influence the St activity and biovailability. Through the oral cavity, Ht gives rise to better supramolecular entities with Pm than with Rs proteins and then can be released in the stomach and adsorbed in the gut.

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