Structure and dynamic studies by NMR of the potent sweet protein monellin and a non-sweet analog

Evidence on the importance of residue Asp^{B7} for sweet taste

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Abstract Monellin, an intensely sweet protein and a non-sweet analog in which the Asp^{B7} in monellin has been replaced with Abu^{B7} were studied by NMR. The results of our investigations show that the 3-dimensional structure of these two proteins are very similar indicating that the lack of the β-carboxyl group in the Abu^{B7} analog is responsible for the loss of sweet potency. Selectively labeled monellin was prepared by solid-phase peptide synthesis by incorporating ^{15}N-labeled amino acids into 10 key positions including Asp^{B7}. The internal mobility of these 10 key residues in monellin was estimated by the method of model-free analyses and our NMR studies show that Asp^{B7} is the most flexible of these 10 residues. The flexibility of the Asp^{B7} side chain may be important for receptor binding.
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Key words: Monellin; Solid-phase synthesis; Active residue Asp^{B7}; Selectively ^{15}N-labeled monellin; Model-free analysis

1. Introduction

Monellin was isolated from the West African berries *Dioscoreophyllum cumminsii*. It is several thousand times more potent in sweetness than sucrose on a weight basis [1,2] and consists of two non-covalently associated polypeptide chains, A and B, with 44 and 50 amino acid residues, respectively [3,4]. The X-ray crystal structure of natural monellin [5,6] shows that its tertiary structure consists of an anti-parallel β-sheet with five strands and an α-helix. ^{1}H resonance assignments and determination of the secondary structure and general topology in solution have been carried out for single-chain monellin which was made by combining the N-terminus of the A-chain with the C-terminus of the B-chain, and tastes as sweet as natural monellin [7].

To determine the active site of monellin, a number of analogs were prepared by the solid-phase method [8,9]. Amino acid residues within potential active sites were replaced with natural or unnatural amino acids. Based upon these studies, Asp^{B7} would fit into the sweetness receptor via ionic interaction, and Ile^{B6} and/or Ile^{B8} on both or either side of the putative active residue would also contribute to the receptor

binding via hydrophobic interaction, thus making monellin sweet. In those studies, there were sharp decreases in sweetness of monellin after replacing Asp^{B7} (on the loop between the β-strand and α-helix of the B-chain) with other amino acids. The aspartic acid residue has been modified to Abu, Gly and some other amino acids. Two analogs, [Gly^{B7}]- and [Abu^{B7}]monellin, led to the complete loss of sweetness. Gly^{B7} and Abu^{B7} have no side chain carboxyl group, suggesting the reason why these analogs were not sweet. Therefore, the active residue for fitting into the receptor was concluded to be Asp^{B7}. In particular, the carboxyl group of the β-position is thought to be essential.

To show that there are no differences in the tertiary structure between [Asp^{B7}]- (native) and [Abu^{B7}]monellin except for the region around residue B7, NMR experiments were carried out. The information on chemical shifts (α and amide protons), NOEs and amide-proton exchange rates are compared for each residue in both proteins. Moreover, the local dynamics of the active residue has been studied by relaxation analyses of selectively ^{15}N-labeled monellin containing 10 labeled residues including Asp^{B7}.

2. Materials and methods

2.1. Sample preparation

Synthesis of natural and [Abu^{B7}]monellin was carried out by solid-phase synthesis in a stepwise fashion according to the previous studies [10,11]. For preparation of the selectively ^{15}N-labeled monellin, ^{15}N-Asp was protected to give Fmoc-Asp(Bu^{t})-OH, and ^{15}N-Gly to give Fmoc-Gly-OH at the first step [12,13]. Synthesis of the labeled monellin was carried out by the same method as that of [Abu^{B7}]monellin [11].

Monellin and [Abu^{B7}]monellin were dissolved in a solution (220 μl) of 95% H$_2$O/5% D$_2$O or 99.9% D$_2$O ($\approx$1.5 mM) and adjusted to pH 3.5. The selectively ^{15}N-labeled monellin was dissolved in 220 μl of 95% H$_2$O/5% D$_2$O at pH 3.5, 4.2 and 5.0 ($\approx$1.5 mM); monellin is stable at a lower pH in such a high concentration. A Shigemi microcell (Shigemi Co. Ltd., Tokyo) was used for the NMR measurements.

2.2. NMR experiments

Homonuclear 2D NMR spectra (DQF-COSY [14], TOCSY [15] and NOESY [16]) of monellin and [Abu^{B7}]monellin were recorded on a Bruker AMX600 spectrometer. For both proteins, the mixing times used for TOCSY experiments were 40 and 70 ms, and that for NOESY was 150 ms. The experiments were recorded in a matrix of 2048(F2)512(F1) data points using 48-50 scans. All data were collected at 300 K for both type molecules. These data were processed on a Bruker X32 data station. To examine the dynamics of the selectively ^{15}N-labeled monellin, we measured the relaxation parameters including T_1, T_2 and {^{1}H}-^{15}N NOE by the method of ^{1}H-^{15}N HSQC [17,18] with gradient sensitivity enhancement on a Bruker DMX600 spectrometer. The acquired data matrix was 1024(F2)80(F1) for the relaxation data with five conditions: pH 3.5 (300 K), pH 4.2 (300 K), pH 5.0 (300 K), pH 3.5 (308 K) and pH 5.0 (308 K). The relaxation

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Table 1
Proton resonance assignments for native monellin at pH 3.5, 300 K

	NH	Hα	Hβ	Others
A-chain				
Ile³	8.43	3.52	0.65	Hγ 0.39/0.39/0.1m, Hδ 0.69m
Lys⁴	9.08	4.34	1.41/1.41	Hγ 1.32/1.32
Gly⁵	7.69	3.57/4.57		
Tyr⁶	8.96	5.47	2.43/2.57	Hδ 7.03, Hε 6.82
Glu⁷	8.98	5.39	1.87/1.87	Hγ 2.38/2.38
Tyr⁸	9.71	5.96	2.73/2.88	Hδ 6.92, Hε 6.75
Gln⁹	9.13	5.45	1.51/1.55	Hγ 1.82/1.82
Leu¹⁰	9.30	5.29	1.60/1.60	Hγ 1.75, Hδ 0.91m/1.04m
Tyr¹¹	9.75	4.97	2.60/2.82	Hδ 6.98, Hε 6.67
Val¹²	9.15	4.77	1.82	Hγ 0.98m
Tyr¹³	9.44	5.56	2.75/3.14	Hδ 6.70, Hε 6.72
Ala¹⁴	9.90	5.10	1.37m	
Ser¹⁵	9.48	4.16	4.07/4.07	
Asp¹⁶	9.32	4.07	3.03/3.07	
Lys¹⁷	8.27	4.57	1.94/1.94	Hγ 1.41/1.41
Leu¹⁸	8.16	4.30	1.97/1.97	
Phe¹⁹	8.97	4.81	1.72/2.02	Hδ 6.69, Hε 6.89
Arg²⁰	8.69	4.93	1.63/1.63	Hγ 1.25/1.25
Ala²¹	8.96	5.35	1.22m	
Asp²²	8.76	6.21	2.65 / 2.95	
Ile²³	9.55	5.29	1.85	Hδ 1.04m/1.30
Ser²⁴	9.55	5.60	3.84/4.12	
Glu²⁵	9.09	5.25	2.29/2.29	Hγ 2.13/2.13
Asp²⁶	8.65	4.79	2.74/2.80	
Tyr²⁷	8.62	4.17	2.43/2.72	Hδ 6.96, Hε 6.88
Lys²⁸	8.44	3.98	1.94/2.04	Hγ 1.47/1.47, Hδ 1.68/1.78
Thr²⁹	8.26	4.37	3.90	
Arg³⁰	7.73	4.19	2.05/2.16	Hγ 1.55/1.60
Gly³¹	8.34	3.65/3.97		
Arg³²	8.40	5.52	0.85/0.85	Hγ 1.18/0.98
Lys³³	8.67	4.85	1.76/1.83	Hγ 1.32/1.44
Leu³⁴	9.51	4.75	2.12/2.12	Hδ 1.05m
Leu³⁵	9.26	4.49	1.73/1.73	Hγ 1.56, Hδ 1.21m
Arg³⁶	7.76	4.63	1.86/1.95	Hγ 1.65/1.65
Phe³⁷	8.75	5.09	2.36/3.71	Hδ 6.78, Hε 5.91, Hη 6.11
Asn³⁸	8.80	5.58	2.64/2.77	
Gly³⁹	8.18	3.55/4.38		
B-chain				
Gly¹		3.95/4.03		
Glu²	8.82	4.30	1.97/2.02	Hγ 2.14/2.27
Trp³	8.20	4.86	3.09/3.09	1NH10.08, 2H7.32, 6H8.20, 4H7.37
Glu⁴	9.51	4.85	2.08/2.18	Hγ 2.46/2.46
Ile⁵	8.58	4.85		
Ile⁶	8.73	4.40	2.09	Hδ 1.92m
Asp⁷	8.28	4.75	2.82/2.82	
Ile⁸	8.20	4.21		
Gly⁹	8.56	4.18/4.38		
Phe¹¹	8.60	4.22	3.09/3.24	Hδ 7.11, Hε 6.73
Thr¹²	7.74	3.75	4.37	Hγ 1.49m
Gln¹³	8.52	4.22	2.15/2.37	Hγ 2.60/2.60
Asn¹⁴	8.03	4.27	2.65/2.82	
Leu¹⁵	7.43	3.86	1.84/1.84	Hγ 1.02, Hδ 0.18m/-0.07m
Gly¹⁶	7.88	3.51/3.51		
Lys¹⁷	7.55	3.09	1.18/1.18	Hγ 0.82/0.82, Hδ 1.58/1.58
Phe¹⁸	7.75	4.35	3.25/3.25	Hδ 7.18, Hε 7.10
Ala¹⁹	8.16	3.63	1.61 m	
Val²⁰	7.82	3.04	2.10	Hγ 0.78m/1.16m
Asp²¹	8.53	4.30	2.58/2.83	
Glu²²	8.29	3.82	1.62/1.62	Hγ 1.87/1.87
Glu²³	8.39	3.85	0.98/0.98	
Asn²⁴	8.78	4.61	2.65/2.95	
Lys²⁵	7.18	4.06	1.91/1.91	Hγ 1.59/1.59
Ile²⁶	7.25	3.86	1.89	Hγ 0.85/0.85/1.00m, Hδ 1.16m
Gly²⁷	8.19	3.74/4.10		
Gln²⁸	6.79	3.95	1.72/1.72	Hγ 1.82/1.98
Tyr²⁹	8.52	4.73	2.34/3.48	Hδ 7.10, Hε 6.70

Table 1 (*continued*)

	NH	Hα	Hβ	Others
Gly³⁰	7.29	3.71/4.09		
Arg³¹	8.48	4.26	1.65/1.77	Hγ 1.47/1.47
Leu³²	9.64	4.83	1.72/1.72	Hγ 1.57, Hδ 0.25m/0.81m
Thr³³	8.38	4.49	3.98	Hγ 1.22m
Phe³⁴	9.42	4.06	2.96/3.08	Hδ 7.03, Hε 7.21
Asn³⁵	8.43	4.78	2.56/2.78	
Lys³⁶	7.11	4.40		
Val³⁷	9.23	5.09	2.05	Hγ 1.22m/1.34m
Ile³⁸	7.82	4.85	2.10	
Arg³⁹	8.28	4.37	1.62 1.87	
Cys⁴¹	9.20	4.98	2.83/2.99	Hγ 2.02
Met⁴²	8.30	5.82	1.87/1.87	Hγ 2.22/2.45
Lys⁴³	9.77	5.68	1.75/1.82	
Lys⁴⁴	9.39	4.75		
Thr⁴⁵	8.58	4.30	3.75	Hγ 0.52m
Ile⁴⁶	8.54	4.04	1.39	Hγ 0.95/0.95/0.79m, Hδ 0.59m
Tyr⁴⁷	8.76	4.83	2.65/2.95	Hδ 6.86, Hε 6.63

data were processed with nmrPipe/nmrDraw (Frank Delaglio, NIH), and peak picking and estimating peak intensities of chosen cross peaks were carried out by PIPP (Daniel S. Garrett et al., NIH) on a Sun Spark 10 workstation.

3. Results and discussion

3.1. Comparison between monellin and [Abu^{B7}]monellin

Signal assignments of monellin and [Abu^{B7}]monellin were made by 2D NOESY, TOCSY and DQF-COSY except for the proline residues and some terminal residues, based on the method of sequence-specific resonance assignment [19] shown in Table 1. The chemical shift differences between both proteins for α protons and amide protons are summarized in Fig. 1. There were chemical shift changes of less than 0.2 ppm between monellin and [Abu^{B7}]monellin with the exception of the α protons at the position of B6, B7 and B8, the amide proton at B6 and the amide proton at B37. The chemical shifts of Ile^{B6} and Ile^{B8} can be rationalized as the direct influence of the amino acid replacement at those adjacent B7. However, the original down-field chemical shift (9.23 ppm) of the amide proton at B37 is extraordinary as an amide proton in a loop region, and the up-field shift by 0.19 ppm upon the replacement suggests a loss of some indirect interaction of Val^{B37} with the carboxylate oxygen at B7. Accordingly, inspecting the crystal structure of monellin [5,6], Arg^{B39} was found to be a probable candidate of the transit residue for this indirect interaction.

The sequential NOEs observed by NOESY experiments for monellin and [Abu^{B7}]monellin (shown in Fig. 2) provide a basis for identifying several secondary structure elements in solution [19]. They show almost the same pattern except the region around B7. We found regions with strong dαN(i,i+1) connectivities, which indicate extended peptide chain conformations, referring to six β-strands. Interstrand NOEs, especially dαα(i,j) and dαN(i,j) connectivities, indicated that these portions of the molecules form an antiparallel β-sheet, as shown in Fig. 3. A series of strong dNN(i,i+1) and weak dαN(i,i+1) connectivities in combination with dαN(i,i+3), dαN(i,i+4), dβN(i,i+1) and dαβ(i,i+3) indicated an α-helical region.
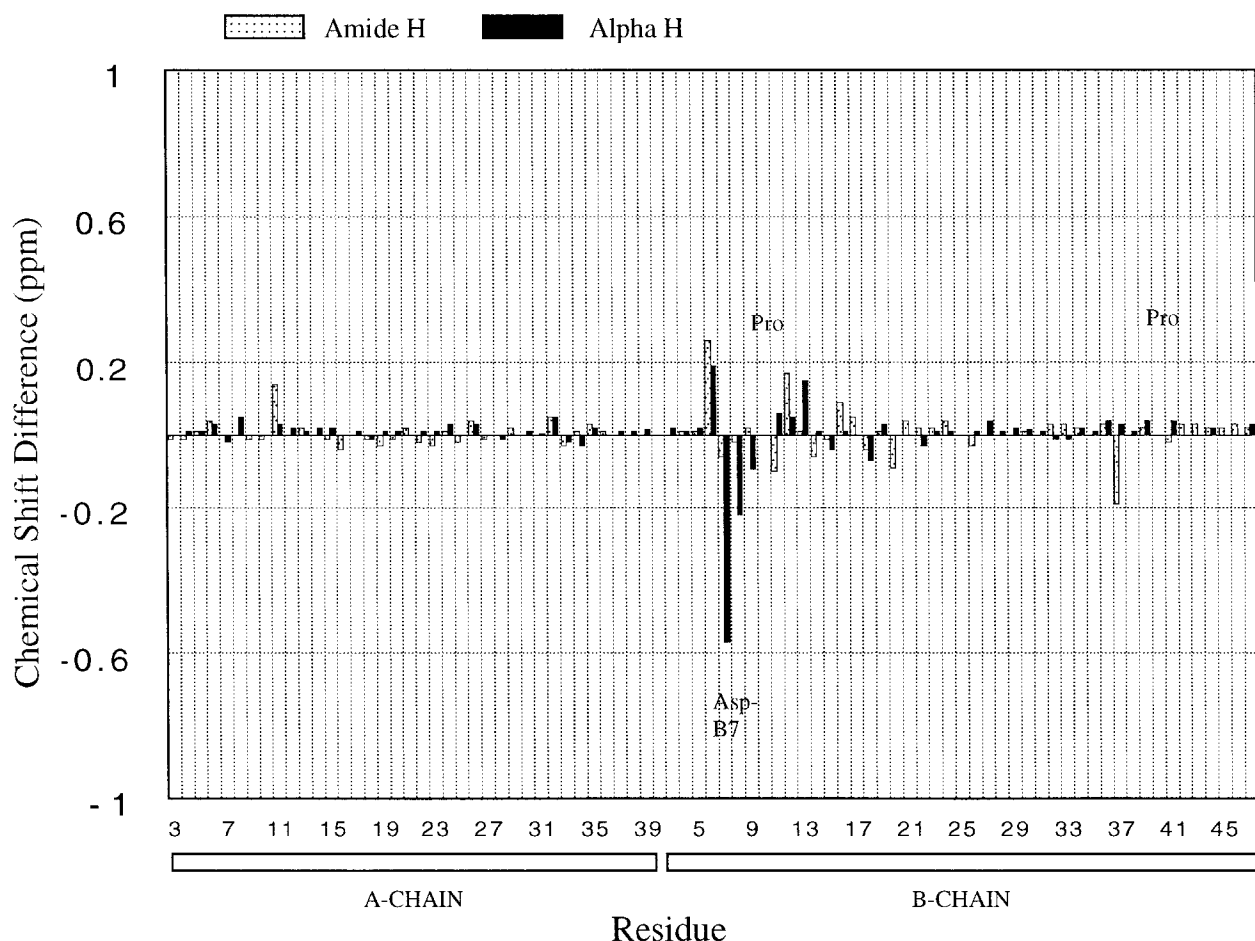
For both proteins, some interchain NOEs between hydro-

Fig. 1. Chemical shift differences of NH (spotted bars) and αH (dark bars) between those in natural and [Abu^{B7}]monellin.

phobic residues were observed, suggesting the existence of a hydrophobic core. The NOE signals were observed between side chains of Ile^{A23} and Ala^{B19}, Ile^{A23} and Phe^{B18}, Ala^{A21} and Phe^{B18}, Ala^{A21} and Phe^{A37}, Leu^{B15} and Phe^{B18}, Tyr^{A8} and Leu^{B15}, Leu^{A10} and Leu^{B15}, Phe^{A37} and Ala^{B19}, and Ala^{A21} and Ala^{B19}. These interchain hydrophobic interactions would contribute to the folding stability of the A- and B-chains with additional interactions of hydrogen bonds between β-strands. Comparison of short- and long-range NOEs and chemical shifts between monellin and [Abu^{B7}]monellin showed no marked differences except for the region of the active residue. Thus, the complete loss of sweetness in [Abu^{B7}]monellin is caused by the lack of the β-carboxyl group in Asp^{B7} and not a result of major disruption in the overall 3-dimensional structure. We therefore conclude that Asp^{B7} is essential for binding to the sweetness receptor.

3.2. Relaxation analyses of the selectively labeled monellin prepared by the solid-phase synthesis

In general, internal flexibility of a protein is important for its biological activity [20]. A region of poor NOEs indicates conformational flexibility or disorder as observed at the N-terminus of the A-chain, the C-terminus of the B-chain, and around the putative active residue. In solution, the results of NOE and amide-proton exchange rate measurements suggested that the range of the β-strand at the N-terminus of the B-chain was shorter and that the active site had more

intramolecular motion than those in the crystal structure. Therefore, we expected to experimentally confirm that the Asp^{B7} residue has higher internal mobility.

NMR relaxation analyses have been useful in examining the dynamics of proteins in solution. Backbone dynamics of many uniformly ^{15}N-labeled proteins, generally prepared with genetic engineering, have been investigated by inverse-detected 2D-NMR spectroscopy [17,18]. Usually, T_1, T_2 and {^{1}H}-^{15}N NOE of ^{15}N nuclei are measured for all backbone residues in the proteins, and the data are analyzed by a model-free approach to determine generalized order parameters (S^2) that provide information on the internal dynamics of proteins. It is interesting to know how flexible the active site is regarding the order parameter as compared with other regions.

By solid-phase synthesis, replacement of the specific residues located in the active sites with other natural or unnatural amino acids is easier, whereas preparing a uniformly ^{15}N-labeled sample is more difficult. Hence, it is expected that the local mobility of the active residue Asp^{B7} could be estimated by comparison with those of some other residues in monellin synthesized by the solid phase method.

Accordingly, ^{15}N-labeled monellin was prepared by the solid-phase method described above, after selecting 10 residues; Asp^{A16}, Asp^{A22}, Asp^{A26}, Asp^{B7}(active residue), Asp^{B21}, Gly^{A5}, Gly ^{A31}, Gly ^{A39}, Gly ^{B16} and Gly^{B30} (shown in Fig. 4), which includes all Asp residues and five out of eight Gly residues in monellin. The signals of ^{1}H resonance did not overlap with
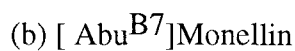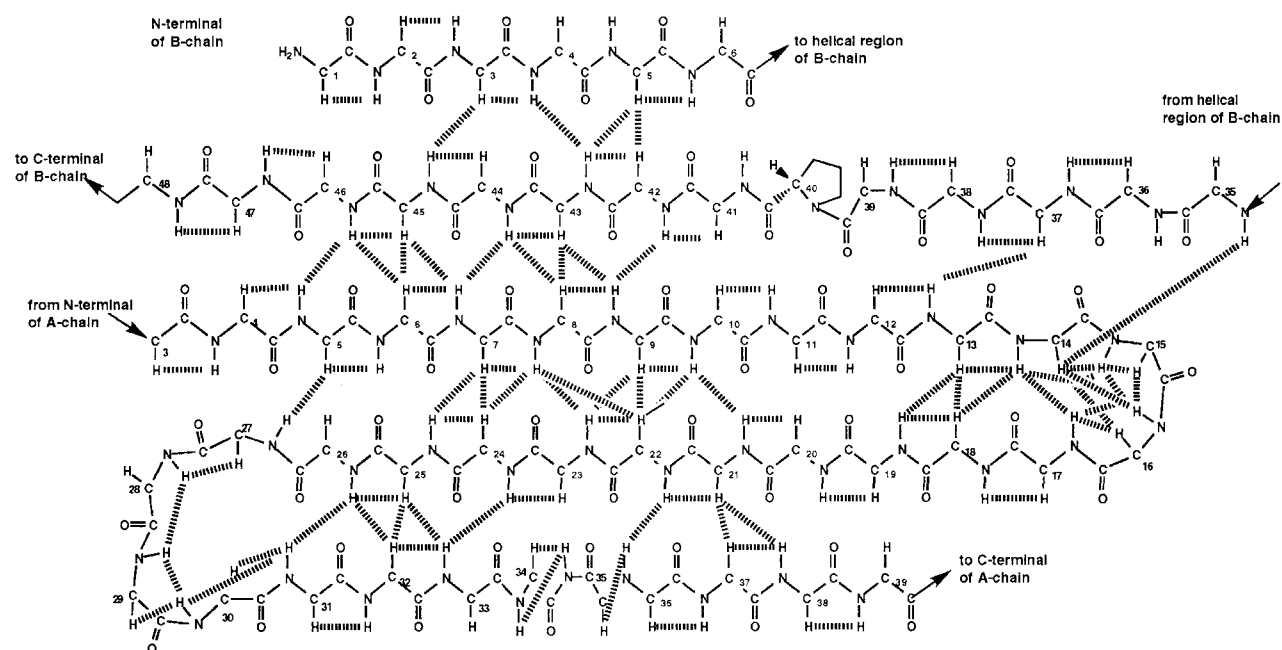
Fig. 2. Summary of NOE connectivities observed for monellin and [Abu[B7]]monellin. Sequential NOEs are represented by bars, and each thickness indicates NOE intensity. Protons that showed slow and intermediate exchange rate are indicated by open circles.
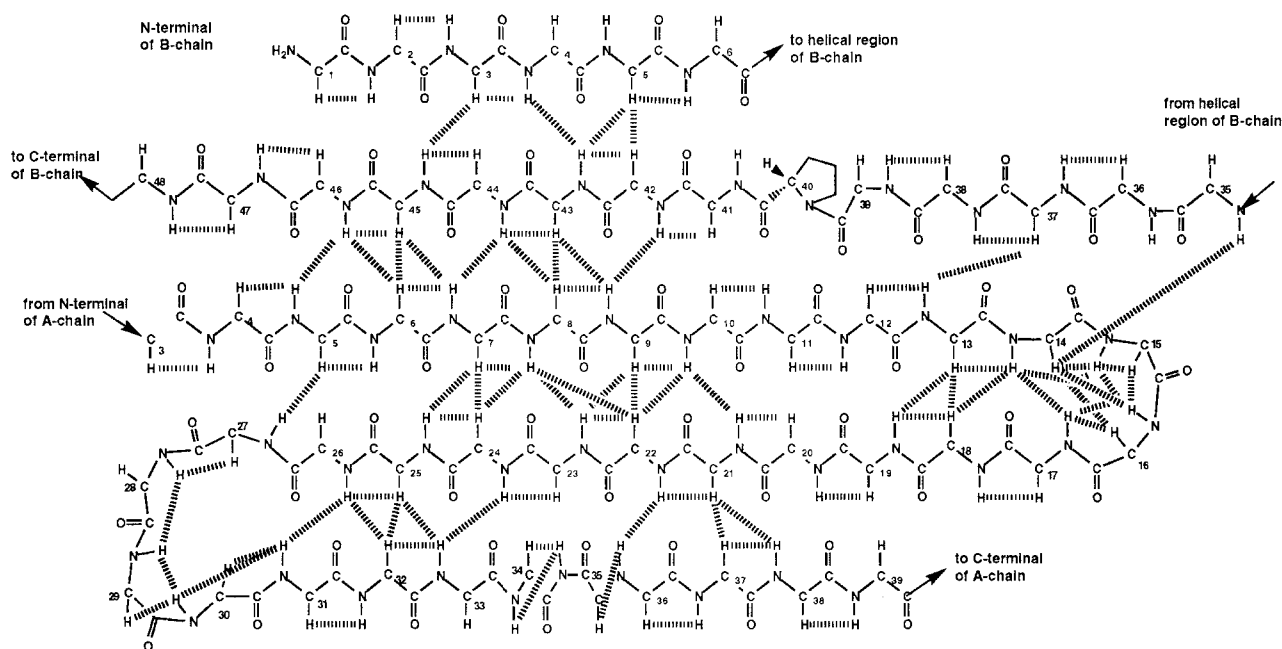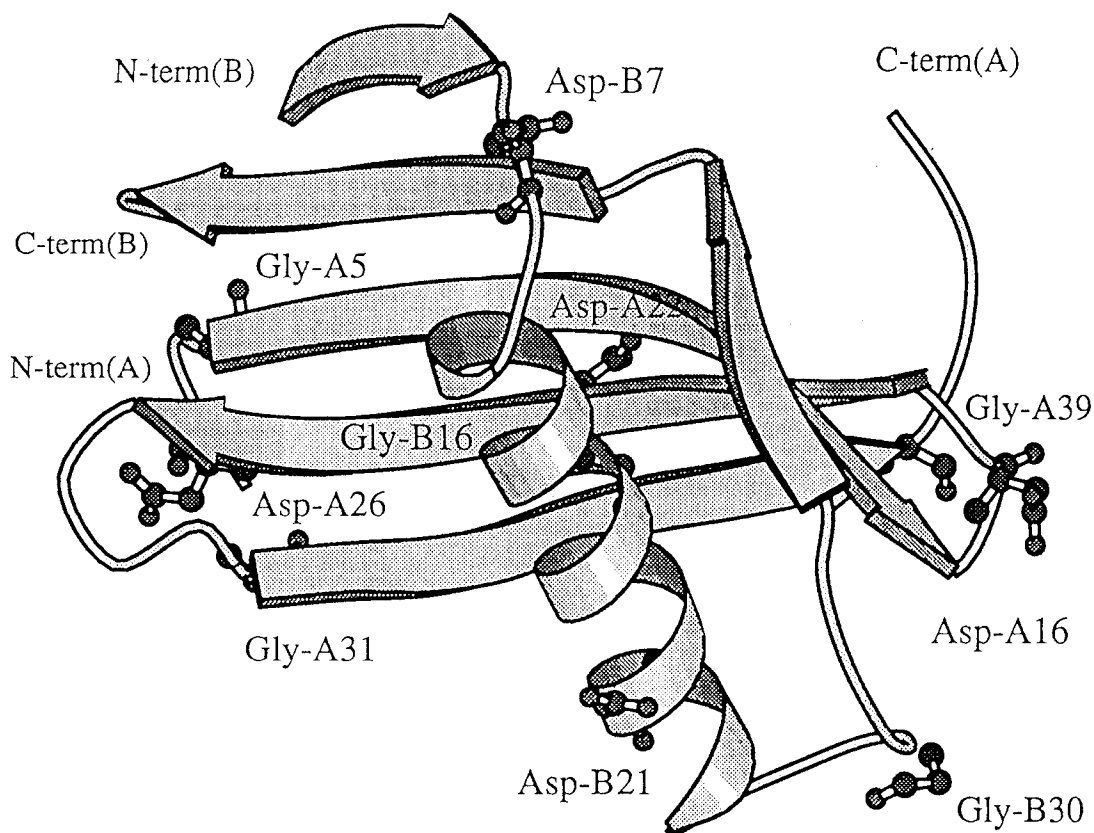
(a) Monellin

(b) [Abu^B7]Monellin

Fig. 3. Short- and long-range backbone NOE patterns indicating the presence of an anti-parallel β-sheet. Observed NOEs are presented by dotted lines.

each other, and they were located at various positions in monellin. The ^{1}H-^{15}N HSQC spectrum is shown in Fig. 5. From these well dispersed signals, the relaxation parameters could be determined more accurately as compared with the case of fully labeled proteins.

The pulse sequences for measurement of relaxation parameters were based on those described previously [17,18]. Relaxation parameters under five conditions (vide supra) were given, in which the tertiary structure was kept [21].

Relaxation parameters of selectively ^{15}N-labeled monellin at pH 3.5 and 300 K are shown in Fig. 6. They corresponded well to the secondary structure of monellin. Asp^{B21} and Gly^{B16} belonging to the α-helix and Gly^{A5} located in the center of the β-sheet showed relatively low internal mobility. The residues on the end of the β-sheet demonstrated higher internal mobility. Asp^{A16} in the turn and Gly^{B30} on the loop also showed little flexibility. The NOEs of dNN(i,j), dαN(i,j) and dαα(i,j) around these residues (shown in Figs. 2 and 3) indi-

N-term(B)   Asp-B7   C-term(A)

C-term(B)   Gly-A5   Asp-A2?

N-term(A)   Gly-B16   Gly-A39

                    Asp-A26

                                                              Asp-A16

Gly-A31

                            Asp-B21

                                              Gly-B30

X-Ray Structure

Fig. 4. Positions of selectively ^{15}N-labeled residues on monellin. X-ray crystal structure (Somoza et al.) is shown [6].

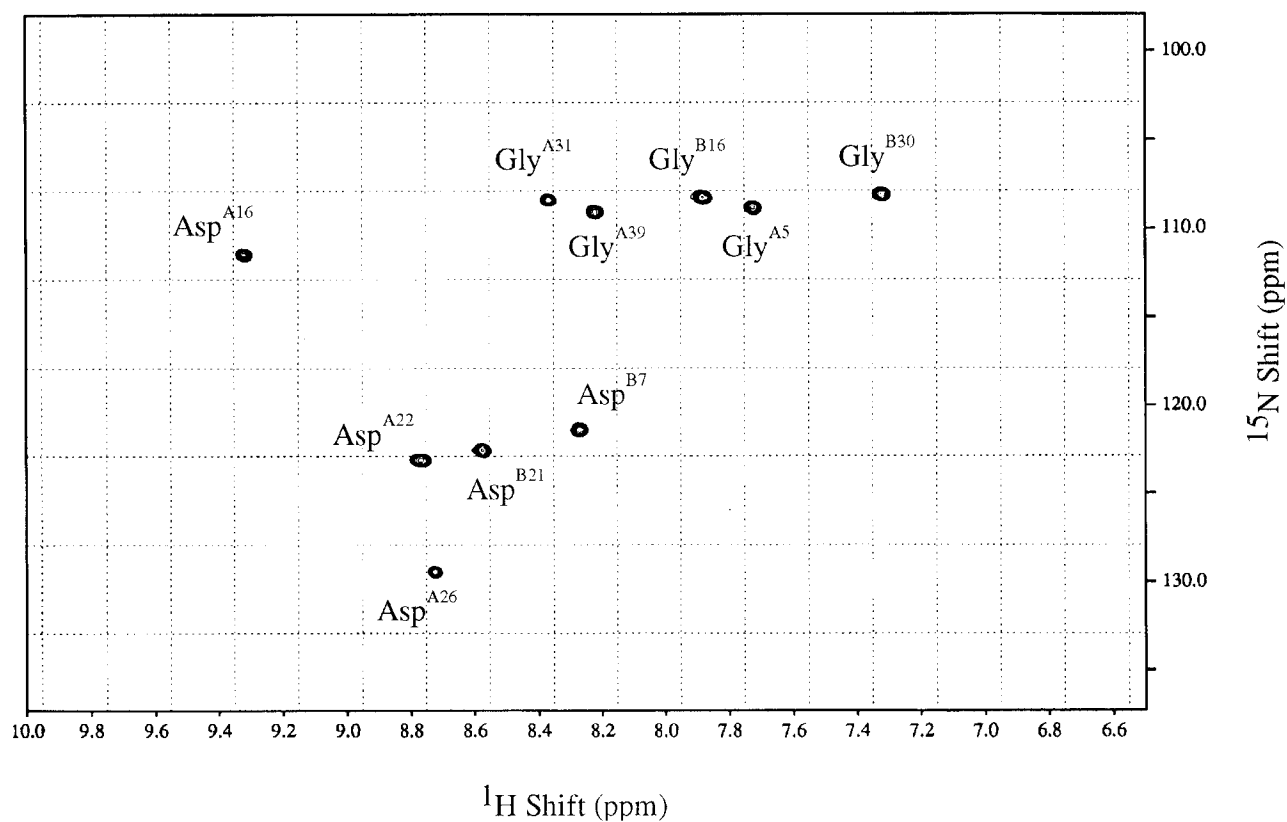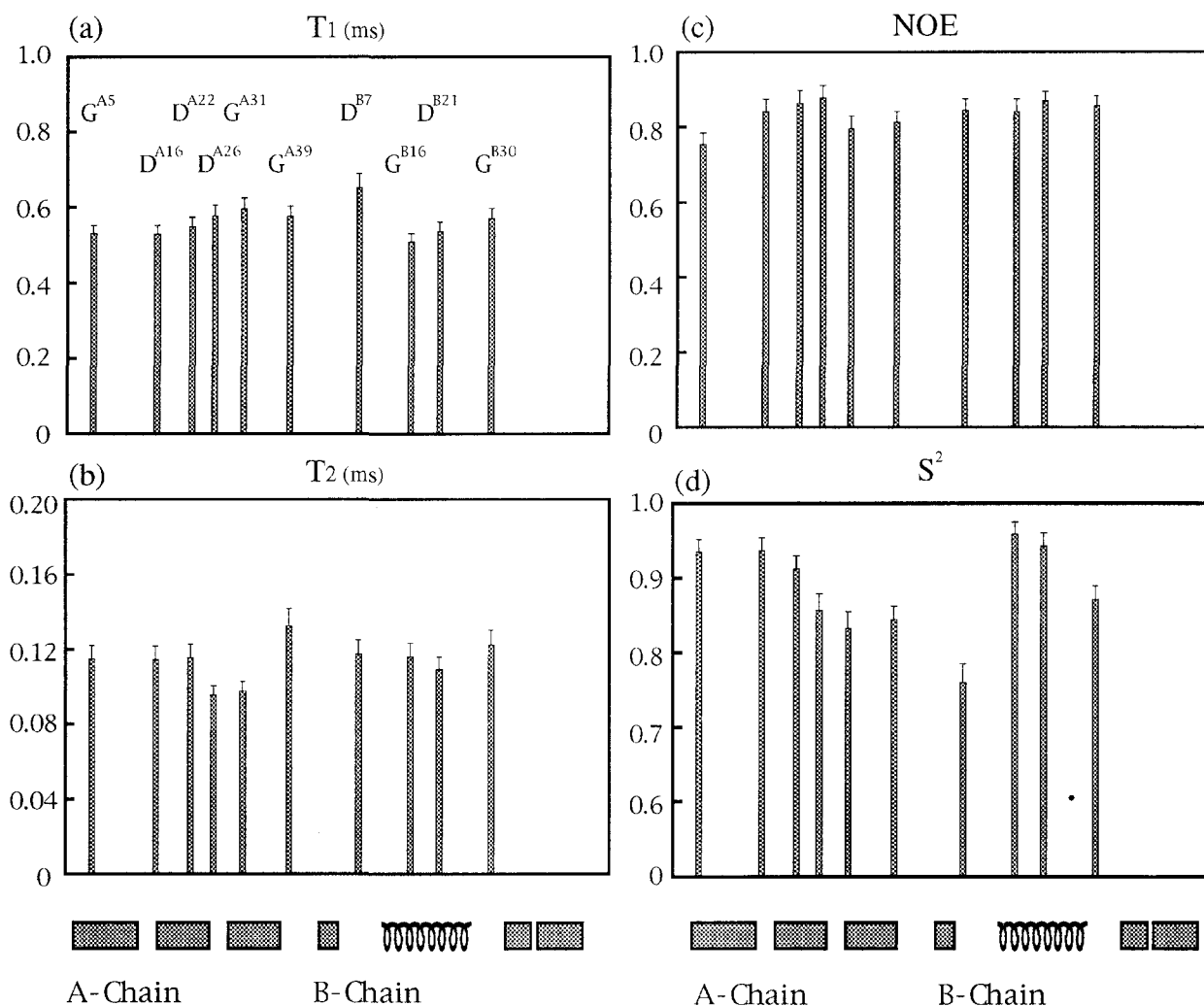Fig. 5. ^{15}N-^{1}H correlation spectrum of 10 residues on labeled monellin is shown.

Fig. 6. ^{15}N-$T1$ (a), $T2$ (b), $\{^1H\}$-^{15}N NOE (c), S^2 (d) values and uncertainties, under the conditions 300 K and pH 3.5, are plotted for 10 labeled residues on monellin.

cated that flexibility of the regions is not always high, and the crystal structure showed the presence of several hydrogen bonds there. In contrast, the Asp^{B7} residue with S^2 (0.76) had a higher flexibility compared to the other nine residues. The value of S^2 estimated in five conditions is shown in Fig. 7. As the temperature rose, the internal mobility became higher for each residue. Furthermore, the value of S^2 for each residue increased with pH. Thus, the whole molecule seemed to be stabilized as a result of ionization in the carboxyl groups of acidic residues around the range of pH 3.5–5.0. For the S^2 value of each residue, a similar change was observed under those conditions, suggesting that partial changes in the tertiary structure were not induced by the changes of hydrogen-ion concentration and temperature within these limited conditions (pH 3.5–5.0, 300 K or 308 K).

The findings of this model-free analyses indicated that the ^{15}N backbone of the active residue Asp^{B7} has a relatively higher flexibility, which is the cause of deficiency of NOE around Asp^{B7}. Furthermore, the chemical shifts of two β-methylene protons in Asp^{B7} showed almost the same values, indicating that the β-position in Asp^{B7} has a higher flexibility. Thus, the active Asp^{B7} residue is concluded to having a higher flexibility both in the backbone and the side chain, making it easy to form the active conformation upon binding to the

sweetness receptor. A general structural model for the sweet taste of small molecules like (R)Ama-(S)Phe-OMe has been proposed, which requires an 'L-shape' structure containing an
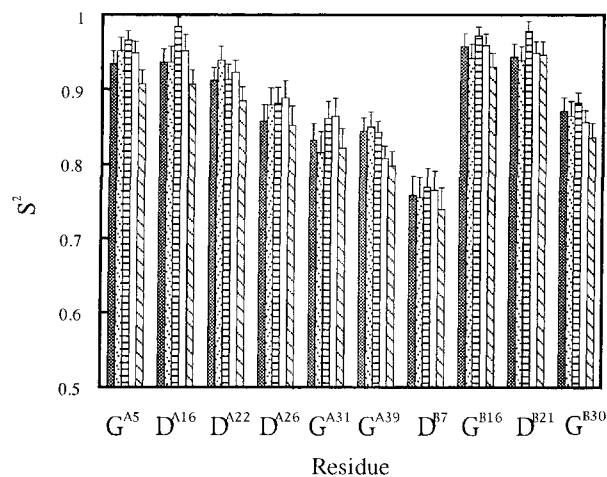

Fig. 7. Values of S^2 recorded under the five conditions of pH 3.5 and 300 K (first bars), pH 4.2 and 300 K (second bars), pH 5.0 and 300 K (third bars), pH 5.0 and 308 K (fourth bars), and pH 3.5 and 308 K (fifth bars).

aminomalonyl moiety as the stem of 'L' and a hydrophobic phenylalanine side chain as the base of 'L' [22]. It consists of a carboxyl group and a hydrophobic group, and both groups are coplanar. In the active site of monellin, the region of -Ile^{B6}-Asp^{B7}-Ile^{B8}- looks similar to that. Thus, we propose that the higher flexibility of the monellin active site is convenient for forming an active conformation such as an 'L-shape' in binding to the receptor.

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