A Sweet-Sensitive Protein from Bovine Taste Buds. Purification and Partial Characterization*

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ABSTRACT: Partial purification and characterization have been performed on a protein, from bovine taste buds, that complexes sugars and saccharin. The protein was purified to homogeneity by ammonium sulfate and molecular sieve fractionation, as determined by thin-layer and disc electrophoresis, ultracentrifugation, molecular sieve chromatography, and cation-exchange chromatography on Cellex-CM. A probable molecular weight of 150,000 was assigned, based on results from ultracentrifugation, molecular sieve chromatography, and amino acid analysis.

An isoionic point of 9.1, calculated from potentiometric titration of the purified protein, was confirmed by thin-layer electrophoresis and was corroborated by generation of a theoretical titration curve based on an amino acid analysis.

A protein, extracted from bovine taste buds, that forms complexes with sugars and saccharin has recently been reported (Dastoli and Price, 1966). It was found that the strengths of the complexes parallel the degree of sweetness of the compounds, and the effect of pH upon formation of the complexes parallels the effect of pH upon sensitivity of taste buds to sweet compounds *in vivo*. The present investigation was undertaken with the objective of partially characterizing this protein in an attempt at elucidation of the mechanism of action.

Materials and Methods

The source, preparation, and assay of the sweet-sensitive protein have been previously described (Dastoli and Price, 1966). The assay method was by refractometry with a Bausch and Lomb Abbe-3L refractometer thermostated at 25 ± 0.01°. The refractometric method of detecting complexes has been used with dilute aqueous solutions of sugars and proteins by Arshid *et al.* (1955). In this method, the refractive increments for the extracts and for the sugars were determined of (i) mixtures of each extract or fraction in the purification procedures with each sugar, and (ii) each fraction and each sugar individually. Derivations from additivity of refractive increments were determined and significantly different changes were the criteria used to determine activity of the various protein fractions. The supernatant from centrifugation at 75,000*g* was subjected to successive ammonium sulfate fractionations. The only fraction showing significant response to sweetness was the one containing material insoluble in 40% (w/v) ammonium sulfate. The data reported are results obtained with this material. Molecular sieves Bio-Gel (Bio-Rad Laboratories) P-30 (50–100 mesh), and P-150 (50–100 mesh) were equilibrated with 0.05 M sodium phosphate buffer (pH 7.0); conditions were (1) Bio-Gel P-30: column 2.5 × 20 cm, flow rate 3.0 ml/min per cm², load 1 ml (20 mg), and temperature 22 ± 2°; (2) Bio-Gel P-150: column 2.5 × 20 cm, flow rate 0.45 ml/min per cm², load 1 ml (20 mg), and temperature 22 ± 2°; and (3) cation exchange: Bio-Rad Cellex CM, column 2.5 × 25 cm, flow rate 1.4 ml/min per cm², eluent 0.01–0.10 M sodium phosphate buffer (pH 6.9), and 0.1–0.5 M by addition of 0.5 M NaOH. Ionic strength changes were determined by conductivity measurements on a Wheatstone bridge. For the disc electrophoresis studies a Canalco Model 12 apparatus (Canal Industries) was employed. The conditions were 6.6 polyacrylamide gel; Tris buffer, 0.1 M, pH 6.6; current 2.5 mA initially and 5 mA after movement; time, 30 min; dye, Naphthol Blue Black; and load, 100 μg. The thin-layer electrophoresis conditions were equilibration vehicle, 0.1 M phosphate buffer (pH 7.0); stationary phase, aluminum oxide-G; 400–450 V, 28–36 mA; time, 1 hr; developer, 2,7-dichlorofluorescein in 0.02% methanol; visualized as purple under ultraviolet light, pink in visible light. Ultracentrifuge studies were carried out in a Model E Spinco ultracentrifuge equipped with schlieren optics. The run was carried out at 5° in a standard cell of Kel-F material at 59,780 rpm: protein concentration, 10.22 mg/ml in 0.1 M phosphate buffer (pH 7.3); time, 75 min. Weight-average molecular weight determinations were performed by the Archibald technique extrapolated to $T = 0$; assumptions, $dn/dc = 0.186$, $\bar{v} = 0.740$; speed, 8225 rpm at 5°. The amino acid analysis was performed on the Beckman Model 120B at 55° in the accelerated run of 4 hr. The digest used was an acidic hydrolysis of the lyophilized protein carried out according to the procedure described by Neurath (1963). Titrations of the protein were performed on a Radiometer titrigraph, type SBR 2e with standardized 0.1 N HCl and 0.1 N NaOH. The protein was titrated both after dialysis,

* From Monsanto Research Corporation, Everett, Massachusetts 02149. *Received June 15, 1967.* Supported by funds from Monsanto Co., St. Louis, Mo.

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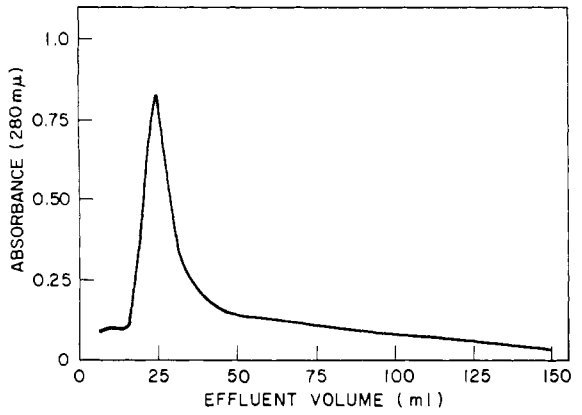

FIGURE 1: Elution pattern of active taste bud fraction on Bio-Gel P-150. Column, 2.5 × 20 cm; flow rate, 0.45 ml/min per cm²; eluent, 0.05 M phosphate buffer (pH 6.9); load, 1-ml (20 mg) active fraction; temperature, 22 ± 2°.

against distilled, deionized water, and in the presence of 0.1 M NaCl. Protein content was determined both by the method of Lowry (Lowry *et al.*, 1951) and by the micro-Kjeldahl method. Nitrogen found was 15.46%. Amide nitrogen was estimated by extrapolation at zero time of the amounts of ammonia produced during the acid hydrolysis. Tryptophan analysis was performed using the method of Goodwin and Morton (1946) and by the method of Opieńska-Blauth *et al.* (1963).

Results and Discussion

Purification. MOLECULAR SIEVE CHROMATOGRAPHY. For the initial purification studies, Bio-Gel P-30, with an exclusion limit of mol wt 30,000 and an operating range of 20,000–50,000, was the resin of choice. The early appearance, within one hold-up volume, its magnitude, and the lack of any other major peak suggested that the majority of the protein in the 40% $(NH_4)_2SO_4$ fraction appeared to be of a molecular weight in excess of 30,000. The presence of a small amount of protein (20%) of <30,000 was evidenced and this material was removed from all future preparations, since it showed no activity when assayed.

With the above information in mind, a molecular sieve gel of higher operating range was employed (Bio-Gel P-150). Figure 1 shows the result of this experiment. It can be seen that only one peak is present. Model reference proteins over a wide range of molecular weights (14,000–186,000) were passed through the column under identical conditions in order to approximate the molecular weight of the isolated protein. An approximate molecular weight may be obtained from a linear correlation between the logarithm of the molecular weight of a protein and the ratio of its elution volume (V) to the void volume (V_0) by the method of Whitaker (1963). Using this method, the molecular weight of the isolated protein was found to be 150,000 ± 3000.

CATION-EXCHANGE CHROMATOGRAPHY. In Figure 2 are the results of an experiment to determine the general type and strength of the charges on the molecular sieve purified protein. The protein was subjected to cation exchange on a Cellex-CM resin in the weak acid form. The column was developed by stepwise gradient elution with an increase in ionic strength as well as pH. The development was monitored for ionic strength with conductivity measurements and for pH with a pH meter. It can be seen from Figure 2 that the protein did not emerge from the column until a concentration of 0.5 M and a pH of above 10 was achieved. The results suggest that the protein is fairly homogeneous and of highly cationic character.

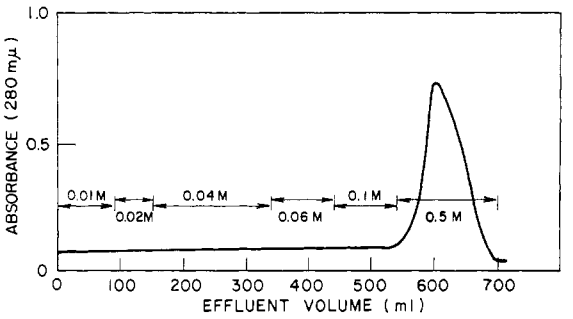

FIGURE 2: Elution pattern of active taste bud fraction on Cellex-carboxymethyl resin. Column, 2.5 × 25 cm; flow rate, 1.4 ml/min per cm²; eluent, 0.01 M sodium phosphate buffer (pH 6.9) to 0.10 M by stepwise addition of 0.10 M sodium phosphate buffer; from 0.10 M sodium phosphate to 0.5 M NaOH by addition of 0.5 M NaOH.

ELECTROPHORESIS. In Figure 3 is a tracing of an electrophoresed alumina thin layer using a method developed in this laboratory (Lopiekes *et al.*, 1966). The active protein, under the conditions stated, migrated cathodally as a single band at 3.5 cm/hr. Its speed and direction of movement are in agreement with those expected from the results of the molecular sieve and cation-exchange chromatography. A 60% $(NH_4)_2SO_4$ fraction, which does not show any affinity for the sugars tested, is also shown in this figure. This fraction moved to a greater extent under the same conditions (8.5 cm) and appears also to be cationic in nature. The small amount of material at 3.5 cm is most likely contamination of the 60% fraction by the 40% $(NH_4)_2SO_4$ com-

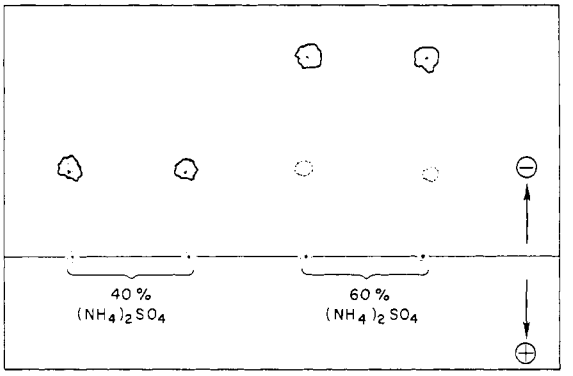

FIGURE 3: Tracing of an electrophoresed alumina thin-layer chromatogram of the column-purified active 40% $(NH_4)_2SO_4$ fraction. Electrophoreses conditions: 0.1 M phosphate buffer (pH 7.7), stationary phase, aluminum oxide G, 400–450 V, 28–36 mA. T = 1 hr. Developer, 2,7-dichlorofluorescein in 0.02% methanol. Visualized purple ultraviolet light. Pink visible light.

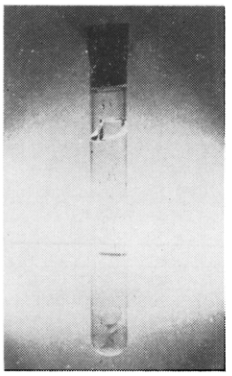

FIGURE 4: Photograph of disc electrophoresis of purified protein. Conditions: 6.6 polyacrylamide gel; Tris buffer (0.1 M, pH 6.6); current, 2.5 mA initially and 5 mA after movement; time, 30 min; dye, Napthol Blue Black; load, 100 μg; direction of migration to the cathode.

ponent. The active protein was also examined by disc electrophoresis (Figure 4). Again, in this case, direction was cathodic and only one band was discernable.

The relative ease of obtaining a homogeneous protein found in these studies is most likely due to the initial Waring Blendor homogenization. The protein is fairly stable to heat and thus survives the heat build-up by this treatment whereas other proteins do not and thus are removed early in the purification procedure.

Characterization. TITRIMETRY. The column-purified active fraction at a concentration of 2.0 mg/ml was dialyzed against distilled, deionized water, diluted 1:100, and titrated in water and 0.1 M NaCl. In Figure 5 are the results of this study. The curve was completely reversible whether run from the acid or alkaline side. This would suggest that no new titratable groups become exposed after treatment at extreme pH values. The curve was essentially the same when performed in either water or 0.1 M NaCl. Inflections appear in the

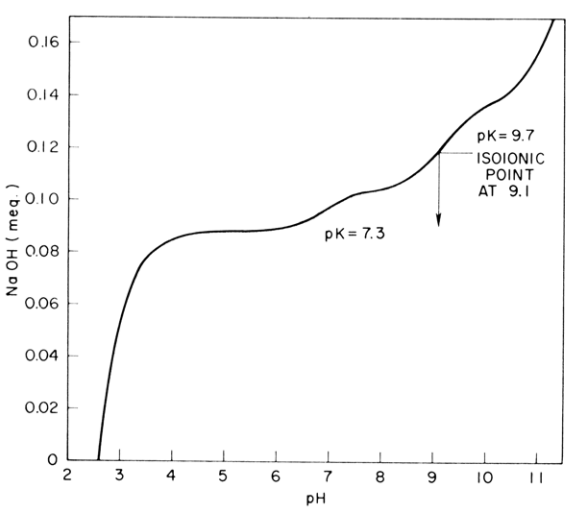

FIGURE 5: Titration of bovine sweet-sensitive protein with standardized 0.1 N HCl and 0.1 N NaOH. The protein (2.0 mg/ml) was dialyzed aginst distilled, deionized water at 4° for 18 hr, and diluted 1:100 before use. Titration in both directions was essentially the same. Calculated isoionic point (pI) was 9.1.

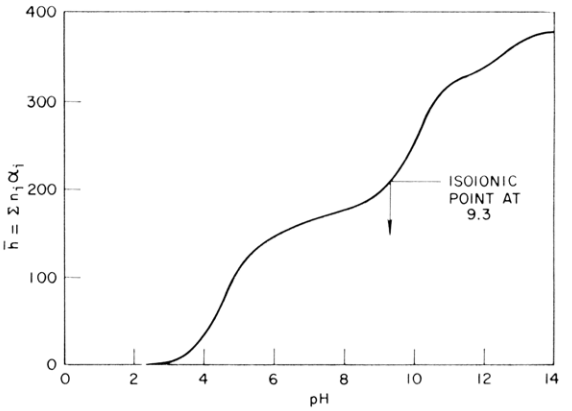

FIGURE 6: Computer-programmed theoretical titration curve of bovine sweet-sensitive protein. The pK values used were taken from Table II. $\bar{n}$ = continuous function of the amount of OH^- ion added.

curve at a pH of 7.3 (minor) and at pH 9.7 (major). An isoionic point was calculated from the data to be 9.1 and indicates an excess of free basic groups. Upon electrophoresis of the protein at pH 9.1, $\Gamma = 0.2$, no net migration was observed. The results of the titration studies support the observations found in the electrophoresis experiments on the cationic nature of the protein. These results would also seem to be in accord with the apparent pH independence of taste receptors as found by us (Dastoli and Price, 1966) and other workers (Beidler, 1962). A pH of higher than 10 would be needed for ionization of the groups present that result in such a high pI value for the protein. Such a high pH is seldom if ever encountered under physiological conditions and the response of the protein to sugars should be fairly level over a wide range of pH. This was found to be the case for the interaction of this protein with fructose over a pH range of 4.8–10.5 (Dastoli and Price, 1966).

ULTRACENTRIFUGATION. The protein was further examined for purity and again for molecular weight by analysis in the ultracentrifuge. A sedimentation velocity run was performed at 59,780 rpm and 5°. Concentration of the protein was 10.22 mg/ml in 0.1 M sodium phosphate buffer (pH 7.3). At 9-min run the sedimenting boundary departed from the meniscus as a single peak and remained as such up to 70 min, whereupon a small shoulder became apparent. A weight-average molecular weight determination by the Archibald technique was performed at 8225 rpm at 5°. Extrapolation to $T = 0$ was made from five pictures taken at 40, 50, 60, 70, and 100 min of the run. The weight calculated was 152,000.

AMINO ACID ANALYSIS. Acidic hydrolysis of the lyophilized, column-purified protein was carried out according to the procedure described by Neurath (1963). The amino acid analysis was performed on the Beckman Model 120B at 55° in the accelerated run for 4 hr. The results of this analysis are presented in Table I. The first column in the table represents the absolute concentrations of each amino acid in the protein in terms of μmoles/0.3 mg.

Minimal molecular weights were first calculated from the weight percentages of the individual acid residues, and the nearest integral numbers for the amino acid

TABLE I: Amino Acid Composition of Sweet-Sensitive Protein.[a]

Amino Acid A	Amino Acid Recov[b] B	Amino Acid Residue/100 g of Protein (g) C	Min Mol Wt (g)[c] D	Nearest Integral No. of Amino Acid Residues for 150,000g Protein (g) E	Integral Nos. of Residues × Residue Mol Wt (g) F
Lysine	0.212	9.05	1,414	106	13,568
Histidine	0.057	2.60	5,269	28	3,836
Amide NH_3	0.353	(1.65)[d]		(177)[d]	(2,478)[d]
Arginine	0.105	5.46	2,857	53	8,268
Aspartic acid	0.261	10.00	1,150	130	14,950
Threonine	0.154	5.18	1,949	77	7,777
Serine	0.175	5.07	1,716	87	7,569
Glutamic acid	0.385	16.55	779	192	24,768
Proline	0.208	6.72	1,443	104	10,088
Glycine	0.207	3.93	1,450	103	5,871
Alanine	0.180	4.26	1,667	90	6,390
Cystine ($^1/_2$)	0.033	1.12	9,107	16	1,632
Valine	0.202	6.66	1,486	101	9,999
Methionine	0.040	1.74	7,528	20	2,620
Isoleucine	0.115	4.33	2,609	57	6,441
Leucine	0.221	8.33	1,356	111	12,543
Tyrosine	0.071	3.86	4,222	36	5,868
Phenylalanine	0.090	4.41	3,333	45	6,615
Total		100.92		1,533	148,803

[a] Total N is 14.87%. [b] The values are expressed as μmoles of amino acid/0.3 mg of protein. [c] Calculated from the relationship (amino acid residue molecular weight) × 100/per cent of amino acid residue in protein. [d] This number is omitted from the total.

residues were obtained for 150,000 g, the molecular weight of the protein suggested by the physical method. A molecular weight of 148,803 g could then be computed as a first approximation from these numbers. A more refined estimation of the molecular weight cannot be justified because of the high molecular weight and the number of amino acids present, in most cases greater than 50 residues/mole of protein. In order to determine the the integral numbers of those amino acids present to an extent of more than 50 residues/mole with certainty, an over-all accuracy of better than 0.5% would be required. Therefore, the only conclusion that can be drawn is that the amino acid composition is consistent with a molecular weight of about 150,000, as determined by physical methods, and that a more exact value for the molecular weight can not be derived from the amino acid analysis.

The sum of the values of column C, representing the amino acid content on a weight percentage basis, show a recovery of 100.9%. This yield of almost 100% is a gross check on the reliability of the experimental results. Furthermore, it would appear that the presence of major components other than the amino acids indicated is unlikely. Repeated tryptophan determinations were inconclusive in assigning a value for this amino acid. The conclusion drawn is that its presence was not sufficient for detection by the methods used.

The experimental results offer the possibility of computing a hypothetical titration curve of the protein and determination of its isoionic point. Because the differ-

TABLE II: Ionizable Groups Available for Titration of Sweet-Sensitive Protein.

Ionizable Group	Total No. in Protein	p*K* Assumed
γ- and δ-Carboxyl	145[a]	4.5
Imidazolium	28	6.5
ε-Ammonium	106	10.0
Phenolic hydroxyl	36	10.0
Sulfhydryl	16	8.5
Guanidinium	53	12.5
End groups	*b*	

[a] Calculated as 322 carboxyl − 177 amide group. [b] End groups were neglected in composing a theoretical titration curve.

ence between the acidic (aspartic plus glutamic amide) and basic (lysine plus histidine plus arginine) is 42 basic groups, one would expect an alkaline isoionic point. A theoretical titration curve for a total of 384 ionizable groups in the protein, neglecting end groups, is shown in Figure 6. An isoionic point of about 9.3 is obtained as compared to the experimental value of 9.1 found in Figure 5. Calculations of the curve were made using a computer program with the assumptions that no electrostatic interaction exists between ionizable groups and that each member of each species is identical. The method of calculation and the pK values used for the individual groups were taken from Edsall and Wyman (1958) and Cohn and Edsall (1943). These values are presented in Table II. Agreement between the theoretical and experimental titration curves is satisfactory, the pI's differing only by 0.2 pH unit. The assumed pK values used may be too high or hydrogen-bond formation among some of the groups could account for this. Comparison of the curves in the range below pH 6.0 would suggest that some masking of groups had occurred in the native protein.

Acknowledgments

We wish to extend our thanks to the following persons for their invaluable aid: Dr. James Schuck, for the initial amino acid analysis; Nick Cirullis, for the ultracentrifugation and analysis; Emery Wescott, for the CAL computer program; and Monsanto Co., for their support for this program.

References

Arshid, R. M., Giles, C. H., McClure, E. C., Ogilvie, A., and Rose, O. (1955), *J. Chem. Soc.*, 67.

Beidler, L. M. (1962), *Progr. Biophys. Mol. Biol. 12*, 107.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides as Ions and Dipolar Ions, New York, N. Y., Reinhold.

Dastoli, F. R., and Price, S. (1966), *Science 154*, 905.

Edsall, J. T., and Wyman, J. (1958), Biophysical Chemistry, Vol. 1, New York, N. Y., Academic, p 477.

Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.

Lopiekes, D. V., Dastoli, F. R., and Price, S. (1966), *J. Chromatog. 23*, 182.

Lowry, O. H., Rosenbrough, N. H., Friar, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Neurath, H. (1963), *Proteins 1*, 33.

Opienska-Blauth, Charaziński, M., and Berbec, H. (1963), *Anal. Biochem. 6*, 69.

Whitaker, J. R. (1963), *Anal. Chem. 35*, 1950.