Evaluation of the Number of Ionogenic Groups of Inulinase by Acid-Base Titration

T. A. Kovaleva, M. G. Holyavka, S. G. Rezvan, and S. V. Kozhedub

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny,* Vol. 145, No. 6, pp. 718-720, June, 2008
Original article submitted November 7, 2007

Acid base titration showed that *Aspergillus awamori* inulinase includes 178 asparaginic and glutamic acid residues, 20 histidine, 10 serine, and 34 lysine and tyrosine residues. Denaturation temperature for this enzyme was calculated using analysis of the proportion of stabilizing and destabilizing amino acids in the molecule.

Key Words: *inulinase; acid base titration*

The study of the molecular mechanisms of functioning of protein molecules, specifically, of enzymes, whose structure remains insufficiently studied, involves quantitative analysis of the content of functional groups determining their physicochemical characteristics and specific functional features. The most available and sufficiently informative method is differential analysis of the buffer capacity of solutions at pH ranges characteristic of dissociation constants of ionogenic groups of amino acid residues. Qualitative amino acid composition of proteins can be evaluated by the increment of buffer capacities and the quantitative proportion of these protein samples can be estimated with high accuracy (up to 1%) by the capacity of functional groups in the protein molecule to reversible dissociation of H^+ ions depending on the microenvironmental pH.

Here we studied buffer capacity and evaluated the number of ionogenic groups of *Aspergillus awamori* inulinase (2,1-β-D-fructane-fructohydrolase, EC 3.2.1.7). This enzyme is highly prevalent in higher plants and microorganisms; it modifies glycoside bonds by cleaving inulin and other fructose-containing polymers to fructose.

MATERIALS AND METHODS

Inulinase isolated from *Aspergillus awamori BKMF 2250* was used in the experiments. The admixtures and ballast substances were removed by staged purification: acetone precipitation, ammonium sulfate fractionation, Sephadex G-25 gel chromatography, and DEAE cellulose ion exchange chromatography. The homogeneity of inulinase fractions was controlled by electrophoresis by modified Davis' method [2]. The characteristics of the resultant enzyme preparation were as follows: 1.5 mg/ml protein, 85.6 U/mg specific activity, 85 purification degree, and 5% output.

Protein was measured by the method of Lowry, catalytic activity of the enzyme was measured spectrophotometrically using Selivanov's reaction [2]. Inulinase activity was calculated by the formula:

$$A = \frac{a}{180bt},$$

where A is catalytic activity (U/mg); a is fructose content (μg); b is the enzyme content in reaction mixture (mg/ml hydrolysate); t is duration of hydrolysis (min); and 180 is the molecular weight of fructose.

Qualitative and quantitative proportions of ionogenic groups in the enzyme molecule were evaluated by the method of acid base titration. Inulinase solutions (10^{-5} mol/liter) were titered by 0.05 n KOH. The increment in buffer capacity (B) caused by dissociation of β- and γ-carboxyl groups (pH

Voronezh State University. ***Address for correspondence:*** tamara_kovaleva@inbox.ru. T. A. Kovaleva

0007-4888/08/1456-0769

2-5), histidine imidazole groups and cysteine SH groups (pH 5-9), and lysine ε-amino groups and tyrosine hydroxyl groups (pH 9-11.5) was evaluated by comparing the data with KCl (0.5 mol/liter) solvent titration curves by the formula:

$$B=\frac{V_{OH}}{V\times m_{OH}},$$

where V_{OH} is total volume of added titered agent in the specified pH interval, V is total volume of titered solution, and m_{OH} is normality of the titered solution.

The amino acid composition of inulinase and number of ionogenic groups were evaluated by the standard methods. The number of SH groups in a molecule was evaluated using p-chloromercuric benzoate, calibration curve was plotted for cysteine. The results were statistically processed using Statgraphics software. The significance of difference was evaluated using Student's t test.

RESULTS

Buffer capacity of inulinase solution at pH 2-5 was 3.5×10^{-23} mol/liter (Fig. 1). The number of asparaginic and glutamic acid β- and γ-carboxyl groups (their dissociation determines buffer capacity) corresponded to 178.0±17.8, the number of histidine imidazole groups was 20±2 (Fig. 2). The resultant number of ionogenic groups is in agreement with the data obtained previously in the studies of amino acid composition of inulinase (Table 1).

The number of cysteine SH groups was 9.50± 0.95, which fully coincided with experimental data of cysteine SH group blocking by p-chloromercuric benzoate and treatment with denaturing agents [3,4].

Buffer capacity at pH 6-9 (pH for histidine imidazole groups and cysteine SH groups) was 0.5×10^{-23} mol/liter. The content of lysine and tyrosine residues corresponded to buffer capacity at pH 9.0-11.5: 0.8×10^{-23} mol/liter (Fig. 2). The number of arginine residues was not determined.

Many-year statistical studies of protein molecules provided ample data on the structural trends of amino acid residues. Amino acids stabilizing and destabilizing secondary structure of proteins were distinguished. Stabilization of individual regulatory structures at the early stage of autoorganization is largely determined by individual characteristics of amino acids [6]. Helix-forming amino acid residues are Leu, Phe, Ile, Met, Val, Lys, Ala, His, Arg, and Ser, while helix destroying ones are Asp, Tyr, Gly, Pro, Cys, and indifferent residues are Gln and Trp [7].

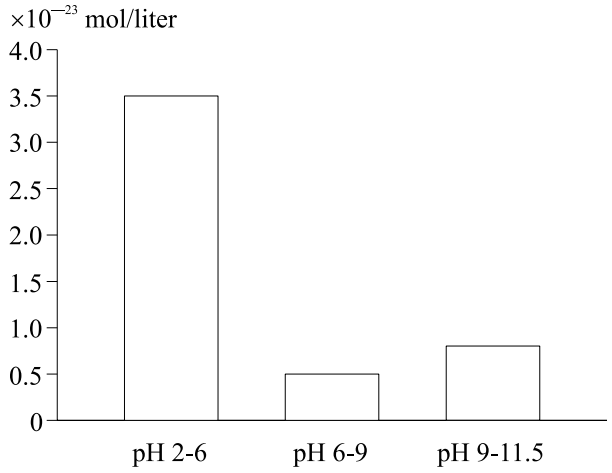

Fig. 1. Buffer characteristics of inulinase solution.

Based on the available data (Table 1), we calculated the temperature of enzyme denaturation (T) by the formula [4]:

$$T=64.5+0.9Y_1-0.6Y_2,$$

where Y_1 and Y_2 is the percent of stabilizing and destabilizing amino acid residues in the protein, respectively.

The total percentage of stabilizing amino acids is 45.39, that of destabilizing ones 46.85, hence,

TABLE 1. Amino Acid Composition of *Aspergillus awamori* Inulinase

Amino acids	Amino acid content in protein, %
Glutamic acid	15.53
Asparaginic acid	11.49
Serine	8.02
Leucine	6.97
Arginine	6.49
Threonine	6.31
Glycine	6.29
Lysine	5.61
Alanine	5.59
Proline	4.92
Tyrosine	4.91
Phenylalanine	4.81
Valine	4.24
Isoleucine	3.77
Histidine	3.38
Cysteine	1.07
Methionine	0.60

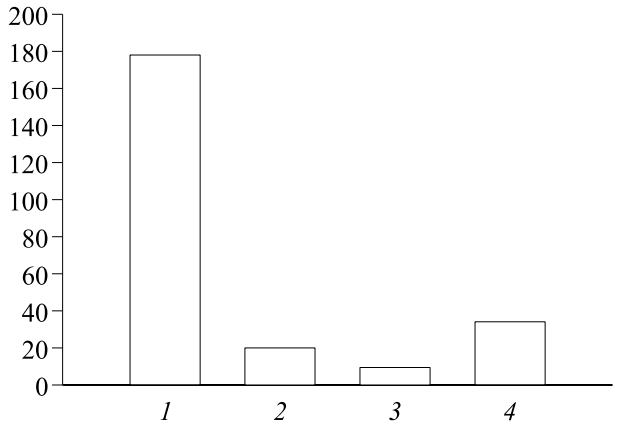

Fig. 2. Number of ionogenic groups in inulinase molecule. *1*) aspartic and glutamic acid carboxyl groups; *2*) histidine imidazole groups; *3*) cysteine SH groups; *4*) lysine NH groups, tyrosine OH groups.

the denaturing temperature is 77°C. These data correlate with experimental data on inulinase thermostability [1].

Hence, *Aspergillus awamori* inulinase is a thermoresistant enzyme. The greatest contribution into the foration of the protein molecule is made by acid amino acids (aspartic and glutamic), which presumably determine stability of the secondary and tertiary structure of this protein.

REFERENCES

1. A. M. Balayan, L. A. Pivazyan, R. N. Khachaturyan, *et al.*, *Biokhimiya*, **61**, No. 5, 895-902 (1996).
2. A. A. Zemlyanukhin and L. A. Zemlyanukhin, *Great Practicum for Plant Biochemistry and Physiology* [in Russian], Voronezh (1996).
3. T. A. Kovaleva, O. M. Kozhokina, and O. D. Trofimova, *Radiats. Biol. Radioekol.*, **40**, No. 1, 23-27 (2000).
4. T. A. Kovaleva, O. M. Kozhokina, and V. F. Selemenev, *Vestn. Voronezh. Gos. Universiteta*, Series Chemistry, Biology, No. 1, 64-67 (2001).
5. T. A. Kovaleva, O. M. Kozhokina, L. A. Bityutskaya, *et al.*, *Sorbtsion. Khromatogr. Protsessy*, **2**, No. 3, 360-366 (2002).
6. A. V. Finkelstein and O. B. Ptitsyn, *Protein Physics* [in Russian], Moscow (2002).
7. T. Creighton, *Protein Folding. Molecular Biology and Biotechnology. A Comprehensive Desk Reference*, New York (1995), P. 754-757.