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Molecular abnormality of human alpha$_1$-antitrypsin variant (Pi-ZZ) associated with plasma activity deficiency

(trypsin inhibitor/pulmonary emphysema/amino acid substitution)

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ABSTRACT **A human alpha$_1$-antitrypsin variant protein was purified to homogeneity from homozygous variant subjects (*Pi-ZZ*) who had a deficiency of plasma trypsin inhibitory capacity. Molecular weight, specific trypsin inhibitory capacity, and immunologic activity of the variant protein were identical to those of normal. Amino acids, *N*-acetylglucosamine, and hexose contents were closely similar in the normal and variant proteins, but the sialic acid content in the variant protein was significantly lower than normal. The structural difference between the normal and the variant alpha$_1$-antitrypsin was elucidated by fingerprinting of their tryptic peptides. Two amino acid substitutions, i.e., glutamic acid in the normal protein to lysine in the variant protein, and glutamic acid in the normal protein to glutamine in the variant protein, were found.**

An inherited deficiency of alpha$_1$-antitrypsin (A$_1$AT) in human serum is associated with pulmonary emphysema and liver cirrhosis (1–4). Of the many genetic variants of A$_1$AT reported, homozygous *Pi-ZZ* has severe serum A$_1$AT deficiency (about 10% of the activity of the normal *Pi-MM* subject). Frequency of the *Pi-Z* gene is high in man, i.e., heterozygous *Pi-MZ* and homozygous *Pi-ZZ* were reported as 4 and 0.02%, respectively (5). Both homozygous *Pi-ZZ* and heterozygous *Pi-MZ* subjects are predisposed to emphysema (3). Thus, the abnormality of the ZZ type of A$_1$AT deficiency draws the attention of many investigators.

It has been observed, by staining the tissues with fluorescent antibody, that *Pi-ZZ* or *Pi-MZ* individuals store globules in their liver that immunologically react with anti-A$_1$AT serum, whereas normal liver does not have such material (6, 7). Recently, the inclusion bodies which were periodic acid–Schiff positive and contained immunologically reactive material were isolated from the liver of *Pi-ZZ* subjects (8, 9). These findings would suggest that the A$_1$AT deficiency in the *Pi-ZZ* variant sera occurs because the variant Z-type of protein (A$_1$AT-ZZ) or its precursor remains in the liver and is not efficiently released into sera (7, 9).

Thus far, our knowledge of the molecular abnormality of variant *A$_1$AT-ZZ* is very limited. Rowley *et al.* estimated that the specific antitryptic activity of variant *A$_1$AT-ZZ* is similar to that of normal *A$_1$AT-MM*. They concluded that the lower activity of *Pi-ZZ* sera is due to its lower concentration of the variant *A$_1$AT-ZZ* (with normal antitryptic activity) and not due to the production of a variant molecule with reduced antitryptic activity (10). However, the specific antitryptic activity of A$_1$AT-ZZ was indirectly estimated, assuming that the immunologic activity of A$_1$AT-ZZ is identical to that of the normal A$_1$AT-MM and disregarding the existence of other plasma proteinase inhibitors, and it remains to be directly measured by means of a purified homogeneous A$_1$AT-ZZ preparation. Based on the observation that the digestion of A$_1$AT-MM sera by neuraminidase produced materials of decreasing anodal electrophoretic mobilities which were similar to those of A$_1$AT-ZZ sera, it was speculated that sialic acid content of variant A$_1$AT-ZZ is presumably lower than that of the normal A$_1$AT-MM (11, 12). Chan and Rees reported the difference of amino acid and carbohydrate content in purified A$_1$AT-MM and A$_1$AT-ZZ (13). However, purity of their A$_1$AT-ZZ preparation and accuracy of the analysis may be argued.

In the work reported here, the variant A$_1$AT-ZZ was purified to homogeneity and the molecular abnormality, i.e., specific amino acid substitutions of the variant protein, was elucidated.

Abbreviation: A$_A$T, alpha$_1$-antitrypsin.

MATERIALS AND METHODS

Normal A$_1$AT-MM was purified to homogeneity from fresh *Pi-MM* blood by slight modification of the method reported by Crawford (14). Trypsin inhibitory capacity was assayed by Eriksson's method (15) with crystalline salt-free trypsin (Worthington Biochemical Corp.) and benzoyl-D,L-arginine-*p*-nitroaniline hydrochloride as its substrate. Soybean trypsin inhibitor (chromatographically purified, Worthington Biochemical Corp.) was used for standardization of trypsin. Acid-starch gel electrophoresis, followed by crossed immunoelectrophoresis, was carried out by a slight modification of Fagerhol's method (16). Electroimmunodiffusion (17) was carried out in 1% agarose gels containing 0.7% (vol/vol) goat anti-A$_1$AT serum (Kallestad Laboratories, Inc.). Protein was determined by the method of Lowry *et al.* with bovine-serum albumin as standard (18). Neuraminidase (*Clostridium perfringens*, lot 55B435) and TPCK-trypsin (trypsin treated with L-tosylamido 2-phenyl ethyl chloromethyl ketone), and carboxypeptidase A and B were from Worthington Biochemical Corp.

RESULTS

Purification of variant A$_1$AT-ZZ

The variant A$_1$AT was purified from fresh blood (ACD anticoagulant) of homozygous variant *Pi-ZZ* subjects. The stepwise procedures are: (*i*) ammonium sulfate fractionation, (*ii*) column chromatography with DEAE-Sephadex, (*iii*) column chromatography with QAE-Sephadex, (*iv*) gel filtration on Sephadex G-100, and (*v*) preparative acrylamide gel electrophoresis. The steps from (*i*) to (*iv*) are similar to Crawford's method (14) which was used for the purification of the normal A$_1$AT-MM, but some modifications were required for the variant A$_1$AT due to the different characteristics of the two proteins. The modifications are as follows. (*i*) Plasma from a unit of blood was mixed with an equal volume of saturated ammonium sulfate and centrifuged; the supernatant was dialyzed against 0.01 M phosphate buffer at pH 7.6.

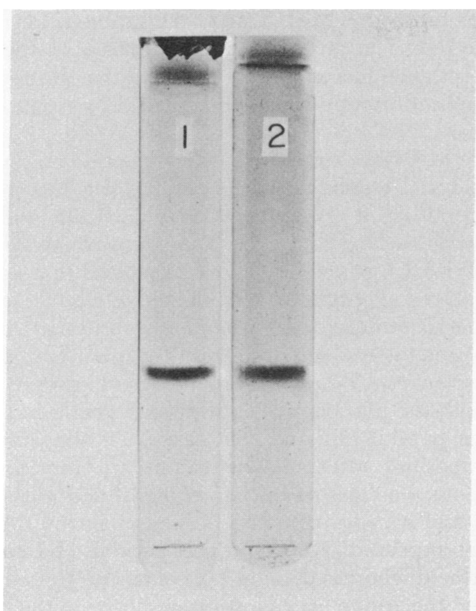

FIG. 1. Acrylamide gel electrophoresis of normal and variant human alpha$_1$-antitrypsin. Electrophoresis was carried out in 7.5% acrylamide gel with Tris-glycerine (23). (1) Normal A_1AT-MM; (2) variant A_1AT-ZZ.

The dialysate was concentrated to about 100 ml by vacuum dialysis and placed on a DEAE-Sephadex column (20 g dry weight, 4.3 × 35cm). (*ii*) After each step of chromatography, the fractions having trypsin inhibitory capacity were pooled and concentrated by vacuum dialysis prior to further purification steps.

Although homogeneous preparation was obtained from normal *Pi-MM* blood after the Sephadex gel filtration step, the preparation of variant A_1AT-ZZ was not homogeneous at this step. Further purification was carried out by preparative acrylamide gel electrophoresis (Poly-Prep from Buchler Instruments) in which a Tris·glycine buffer system as specified by Buchler's instruction manual was used. Starting from 1 unit of variant blood, which contained 70–80 mg of A_1AT-ZZ, about 27 mg of homogeneous A_1AT-ZZ could be obtained. The protein was dialyzed against 0.01 M $(NH_4)HCO_3$ and lyophilized.

Homogeneity of the preparation was demonstrated by acrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate (Figs. 1 and 2).

Electrophoretic pattern of normal and variant A_1AT

Immunoprecipitation patterns on crossed antigen–antibody electrophoresis of crude *Pi-MM* plasma, purified A_1AT-MM, crude variant *Pi-ZZ* plasma, and purified A_1AT-ZZ are shown in Fig. 3. Since both purified preparations had patterns similar to their original plasma, no serious degradation or modification of the protein took place during the purification procedures and the purified A_1AT-MM and A_1AT-ZZ are essentially identical to those existing in the normal and variant blood.

Molecular weight, immunoprecipitation capacity, and specific antitryptic activity

The molecular weight of the variant A_1AT-ZZ is very similar to that of the normal A_1AT-MM because the two proteins migrated equally in sodium dodecyl sulfate-acrylamide gel electrophoresis (Fig. 2). Immunoprecipitation capacity of

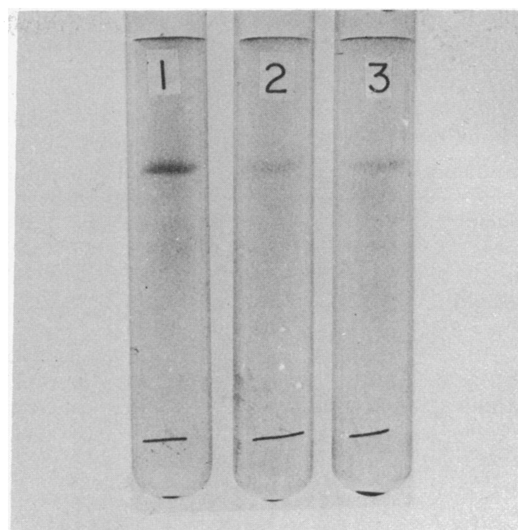

FIG. 2. Sodium dodecyl sulfate-acrylamide gel electrophoresis of normal and variant human alpha$_1$-antitrypsin. Sample preparation and electrophoresis was carried out as described by Weber and Osborn (24). (1) Variant A_1AT-ZZ; (2) normal A_1AT-MM; (3) normal A_1AT-MM digested by neuraminidase.

the purified A_1AT-ZZ, determined by rocket electroimmunodiffusion, is identical to that of normal. The purified A_1AT-ZZ also had the same specific trypsin inhibitory activity as the purified normal A_1AT-MM, i.e., 0.57 ± 0.02 mg of trypsin was inactivated by 1 mg of both proteins.

Amino acid and carbohydrate composition

Amino acid and carbohydrate composition of the normal and variant A_1AT are very similar. Glucosamine and hexose contents of the two proteins are also identical. However, the content of sialic acid in the variant A_1AT-ZZ is significantly lower than that of the normal A_1AT-MM (Table 1).

Amino acid substitution

Amino acid substitution between the normal and the variant A_1AT was examined by peptide mapping of their tryptic digests. Because the two proteins contained different amounts of sialic acid, special caution was taken prior to tryptic digestion. A_1AT-MM and A_1AT-ZZ (about 20 mg each) were digested by neuraminidase (0.7 mg, 6.7 units) in 0.05 M acetate buffer at pH 5.0 for 4 hr at 37°. Release of sialic acid from the protein was followed by assay of free sialic acid in the incubation mixture by Warren's method (19). After 1 hr about 90% and after 4 hr nearly 100% of the sialic acid was released from the protein. Proteolytic enzymes, which might be contaminated in the neuraminidase preparation used, could hydrolyze peptide bonds of the protein. If this would happen during the above treatment, the subsequent structural comparison by peptide mapping of their tryptic digests becomes practically impossible. In order to make certain that cleavage of the peptide bonds did not occur during digestion, we examined the protein digested by neuraminidase by sodium dodecyl sulfate-acrylamide electrophoresis. Since no peptides with smaller molecular sizes were detected, the peptide bonds of the protein remained intact (Fig. 2).

After the neuraminidase digestion for 4 hr, the protein was reduced and *S*-carboxymethylated in the presence of 8 M urea at pH 8.6, as previously described (20). The protein solution was acidified to pH 3 with acetic acid, dialyzed against water, and lyophilized. The *S*-carboxymethylated

Table 1. Amino acid and carbohydrate content of normal and variant human alpha$_1$-antitrypsin

Component	Normal MM		Variant ZZ percent of residues*
	Percent of residues*	Residues per mole†	
Aspartic acid	8.85	40	9.18
Threonine‡	5.65	29	5.54
Serine‡	3.57	22	3.70
Glutamic acid	13.02	53	13.23
Proline	4.68	25	4.84
Glycine	2.27	21	2.35
Alanine	3.23	24	3.54
Half cystine§	0.27	2	0.32
Valine¶	3.92	21	3.96
Methionine	2.15	9	1.95
Isoleucine¶	3.51	16	3.41
Leucine	11.15	52	11.08
Tyrosine	1.94	6	1.97
Phenylalanine	7.85	28	7.62
Tryptophan‖	0.59	2	0.63
Lysine	7.31	30	7.53
Histidine	2.85	11	2.72
Arginine	1.82	6	1.79
Acetylglucosamine**	5.55	13	5.94
Hexose††	6.18	17	6.00
Sialic acid‡‡	3.64	7	2.70
Total	100	434	100

Amino acid analysis was accomplished with an automatic amino acid analyzer (JEOL-6AH) using standard two-column method.

* Except where noted, the value is the average of the 20, 44, and 68 hr of hydrolysis in 6 M HCl at 110°. Total of individual residues is taken as 100.

† The nearest integral number of components per molecule based on molecular weight of 53,000, which was estimated by sodium dodecyl sulfate-acrylamide gel electrophoresis described by Segrest and Jackson (27).

‡ Values extrapolated to zero hydrolysis time.

§ Determined as cysteic acid in performic acid-oxidized protein.

¶ Values of hydrolysis for 68 hr.

‖ Estimated by the method of Goodwin and Morton (25).

** Values of hydrolysis for 5 hr in 3 M HCl at 100°.

†† Estimated by the orcinol reaction (26).

‡‡ Estimated by the thiobarbituric acid reaction (19).

protein was subjected to tryptic digestion in the presence of 2 M urea with the pH of the reaction mixture maintained at pH 8.0 with 0.01 M $(NH_4)_2CO_3$ at room temperature overnight. After eliminating urea by Sephadex G-15 gel filtration, the digests were lyophilized.

Tryptic hydrolysates (4–5 mg of lyophilized powder) were applied to Whatman 3MM paper and separated by the method described by Katz *et al.* (21). Five sets of peptide maps of the normal and variant proteins were prepared. One of them is shown in Fig. 4.

Among the peptide spots appearing in each peptide map, two peptide spots (marked M-1 and M-2) of the normal protein were consistently replaced by two peptide spots (marked Z-1 and Z-2) in the variant protein.

There seemed to be a slight difference in several peptide spots located in the lower left part of the peptide maps when the normal and the variant in this particular set of peptide maps (Fig. 3) were compared, but the difference was not consistently found in other sets of maps, and it cannot be considered real peptide substitution.

The peptide spots marked M-1, M-2, Z-1, and Z-2 were cut out, eluted with 6 M HCl, and their amino acid composition analyzed. The amino acid composition of these four peptides is shown in Table 2. It is evident that glutamic acid (or glutamine) in peptide M-1 is replaced by lysine in peptide Z-1.

Peptide M-1 was extracted from the peptide map with 5% acetic acid and lyophilized. The peptide was digested with carboxypeptidase B (10 units) overnight at 25°, and subsequently with carboxypeptidase (5 units) overnight at 25° in 0.05 M Tris-HCl at pH 8.0. The amino acids that appeared were analyzed; Peptide M-1 contained glutamic acid, not glutamine. Therefore, the amino acid substitution between the two proteins was glutamic acid (normal A_1AT-MM) to lysine (variant A_1AT-ZZ).

Although the two peptides moved differently in the peptide maps, peptide M-2 and peptide Z-2 had the same amino acid composition after acid hydrolysis. In order to resolve the problem, we digested the two peptides with carboxypeptidase B and carboxypeptidase A, and analyzed the amino acids that appeared. We found that peptide M-2 contained glutamic acid whereas peptide Z-2 contained glutamine. Accordingly, the amino acid substitution between the two proteins was glutamic acid (normal A_1AT-MM) to glutamine (variant A_1AT-ZZ).

DISCUSSION

The content of sialic acid in the variant A_1AT-ZZ was lower than that of normal A_1AT-MM, but there was no significant difference in amino acids, hexose, and hexosamine content between the two proteins (Table 1). Chan and Rees reported that their purified A_1AT-ZZ contained larger amounts of arginine and glycine and lower amounts of lysine and threonine (13). The discrepancy could be due to the degree of accuracy of analysis and/or the difference of homogeneity of the variant A_1AT-ZZ preparations. About 35 peptide spots were visible in the fingerprint of the normal and variant human alpha$_1$-antitrypsin. From the number of peptide spots and the content of lysine and arginine residues in the protein, one can be reasonably certain that nearly all the trypsin-susceptible peptide bonds of the neuraminidase-treated S-carboxymethylated protein were hydrolyzed under the conditions used, and that the resolution of the fingerprint was high enough to separate nearly all tryptic peptides.

From the amino acid composition, peptide MM-1 should be (Asx, Thr, Ala, Val, Ileu, Leu)-Glu-Lys and peptide ZZ-1 should be (Asx, Thr, Ala, Val, Ileu, Leu)-Lys-Lys. Accordingly, the amino acid substitution between the two peptides is glutamic acid (normal) to lysine (variant). This amino acid substitution can be attributed to a single step base transition from $\frac{G}{C}$ to $\frac{A}{T}$ in the gene.

Another amino acid substitution found between the two proteins is glutamic acid (normal A_1AT-MM) to glutamine (variant A_1AT-ZZ). This amino acid substitution may be attributed to a single step base transversion $\frac{C}{G}$ to $\frac{G}{C}$ in the gene, but the substitution may also be induced by the post-translational modification of the glutamine residue by transglutaminase (22). Because it is not very likely that two independent base substitutions occurred in the single gene, the substitution from glutamic acid to glutamine is presumably induced by the post-translational modification, rather than by a base substitution in the gene.

The variant ZZ protein contains a lesser quantity of sialic acid than does the normal MM protein. The content of carbohydrate in the protein cannot be directly coded by the nu-

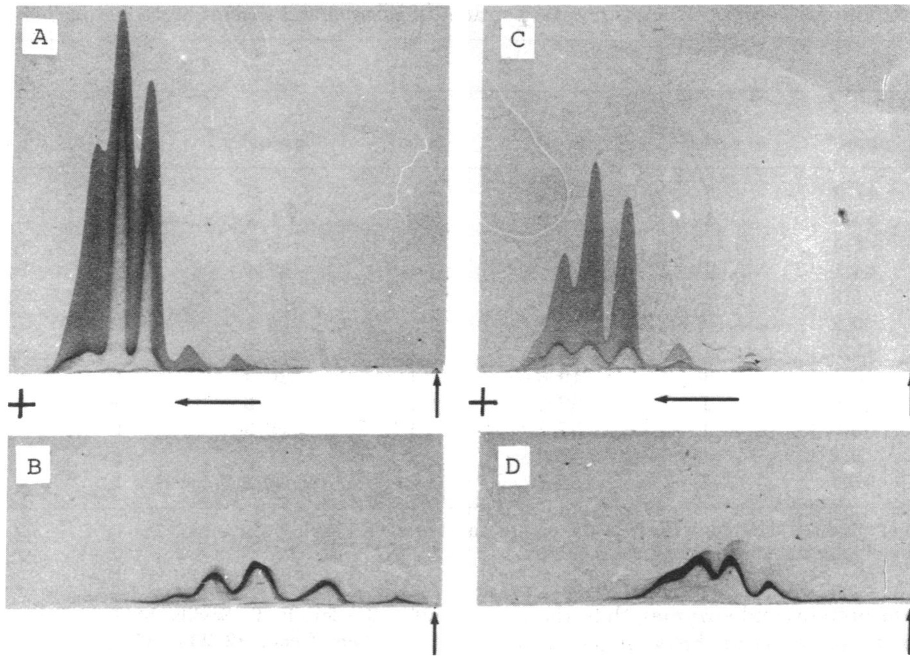

FIG. 3. Immunoprecipitation patterns after crossed antigen-antibody electrophoresis. (A) Crude normal Pi-MM plasma; (B) crude variant Pi-ZZ plasma; (C) purified normal A_1AT-MM; (D) purified variant A_1AT-ZZ. Arrows indicate origins where the sample was applied.

cleotide sequence of mRNA; therefore, the altered sialic acid content is due to a secondary consequence of the amino acid substitution. Because none of the carbohydrate-binding amino acid residues, i.e., asparagine, threonine, and serine, are substituted by another amino acid in the variant protein, the decrease of sialic acid content in the variant protein should be indirectly induced by the change of protein configuration.

Despite its structural abnormality, the specific trypsin inhibitory activity and the immunologic activity of the variant A_1AT-ZZ molecule are identical to that of the normal A_1AT-MM. Consequently, lower plasma antitryptic activity (10–15% of normal in homozygous *Pi-ZZ*) of the variant subjects results from a reduction in the number of variant A_1AT-ZZ molecules in plasma.

The possible mechanisms for the reduction of the number of A_1AT molecules in the variant plasma are: (*i*) less efficient secretion (or activation) of the variant A_1AT (or its precursor) from tissues into plasma; (*ii*) a decreased rate of production; (*iii*) an increased degradation of the variant protein; and (*iv*) a combination of these effects.

An accumulation of an immunologically crossreactive material (6–9) in the variant's hepatocytes favors the first possibility. Reduction in the rate of synthesis is frequently associ-

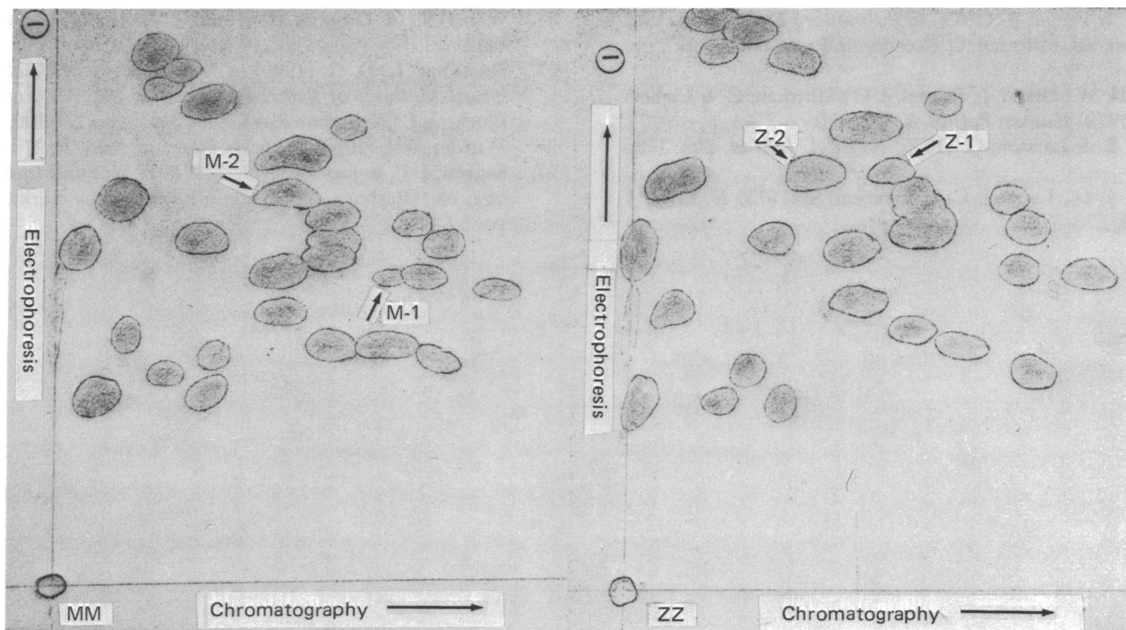

FIG. 4. Peptide maps of tryptic digests of normal and variant human alpha$_1$-antitrypsin on Whatman 3MM paper. First dimension (chromatography): 1-butanol-acetic acid-water (4:1:5) for 18 hr. Second dimension (electrophoresis): pyridine-acetic acid-water (1:10:289, pH 3.6), 2000 V for 80 min. *Left*, normal A_1AT-MM; *Right*, variant A_1AT-ZZ.

Table 2. Amino acid composition of tryptic peptides of normal and variant human alpha$_1$-antitrypsin

	M-1		Z-1		M-2		Z-2	
Amino acid	nmol*	Molar ratio†	nmol*	Molar ratio†	nmol*	Molar ratio†	nmol*	Molar ratio†
Aspartic acid	47.2	1	51.2	1	3.0	—	2.4	—
Threonine	55.6	1	41.9	1	1.3	—	1.8	—
Serine	1.4	—	1.6	—	5.8	—	2.6	—
Glutamic acid	44.1	1	2.4	—	27.4	1	39.7	1
Proline	1.3	—	4.2	—	24.9	1	25.8	1
Glycine	3.2	—	6.3	—	18.2	1	35.2	1
Alanine	40.6	1	50.0	1	1.5	—	<1	—
Valine	46.1	1	49.6	1	20.0	1	33.1	1
Isoleucine	43.6	1	52.2	1	1.6	—	1.2	—
Leucine	59.8	1	57.7	1	16.3	1	26.8	1
Phenylalanine	2.2	—	3.6	—	21.3	1	27.2	1
Lysine	50.1	1	95.6	2	33.2	1	41.8	1

* Mean value of amount of amino acid found in the peptides eluted from two sets of each fingerprint.
† Closest integral number of amino acid residues in the peptides.

ated with many variant proteins and enzymes; thus, the second possibility cannot be excluded in the A$_1$At deficiency. Although the stability of the variant A$_1$AT-ZZ *in vitro* is not different from that of normal, the variant protein could be more rapidly degraded *in vivo*. Therefore, these possibilities remain to be examined.

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