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Stability of recombinant human alpha-1-antitrypsin produced in rice in infant formula[1]

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Abstract

Human milk contains several biologically active proteins that benefit the breast-fed infant. In order to survive in the gastrointestinal tract, these proteins need to be protected against proteolysis. Since human milk contains relatively high concentrations of α-1-antitrypsin (AAT), we have expressed recombinant AAT in rice to explore the possibility of supplementing infant formula with this protein. The stability of recombinant AAT was examined by biochemical and functional assays, such as SDS-PAGE, Western blotting, ELISA, elastase and trypsin inhibition, following exposure to heat, low pH, and *in vitro* digestion, conducted in both phosphate buffered saline and infant formula. Native AAT is resistant to acidic environments down to pH 2 for 1 h and can withstand *in vitro* digestion modeled after conditions in the infant gut. Recombinant AAT is nearly as resistant as the native form in buffer, and is equally resilient in formula. Heat treatments (60°C for 15 min, 72°C for 20 sec, 85°C for 3 min, and 137°C for 20 sec) revealed that recombinant AAT is not as stable as native AAT in buffer, particularly at higher temperatures. While significantly less recombinant AAT is detected by ELISA after heating in formula, addition of bile extract can restore epitopes resulting in higher concentrations, suggesting protein aggregation that may not affect AAT activity. This study shows that recombinant AAT may survive the conditions of the infant stomach and duodenum and affect protein digestion in the infant small intestine.

Keywords: α-1-antitrypsin; Infant formula; Rice; Recombinant human milk proteins; Milk; Infant digestion

1. Introduction

It is generally agreed that breast-feeding is the optimum method for feeding infants. While infant formula can supply nutrients at levels similar to or higher than breast milk, formula-fed infants have a higher prevalence of infection, longer duration of diarrhea and upper respiratory infections, and different growth patterns than breast-fed infants [1-3]. Recent studies have shown that this difference is significant even in affluent socio-economic environments [4].

Breast milk contains biologically active milk proteins currently not available in formula. While different protein sources can be used in formulas to provide amino acid concentrations and ratios similar to human milk, they currently do not contain bioactive proteins. Examples of such milk proteins are lactoferrin and lysozyme, which have bacteriostatic and bactericidal activity [5]. The survival of such proteins is possible due to the immaturity of the newborn infant gut, which features low gastric acid secretion and proteolytic enzyme production [6], resulting in incomplete proteolysis. Some of these proteins, such as lactoferrin and lysozyme, also show an inherent resistance to proteolysis [7,8], most likely related to the structure of the proteins and their glycosylation. Another major factor that may prevent the digestion of these milk proteins is the presence of protease inhibitors, such as α-1-antitrypsin (AAT) in human milk.

AAT belongs to the class of serpin inhibitors, has a molecular mass of 52 kD, and contains about 15% carbohydrate [9]. The concentration of AAT in human milk ranges from 0.1 to 0.4 g/L, and it appears to decrease with infant age [10-13]. While the binding affinity of AAT is highest for human neutrophil elastase, it also has affinity for pancreatic proteases such as chymotrypsin and trypsin [14]. Milk AAT has a molecular weight similar to that of to

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serum AAT and can bind trypsin and chymotrypsin (Chowanadisai and Lönnerdal, unpublished observations). The role of AAT in milk is not well understood, but it is possible that AAT may prolong the survival of other milk proteins through inhibition of pancreatic proteases and allow a proportion of other bioactive milk proteins to survive and affect infant development. In support of this function, AAT can prevent the digestion of lactoferrin by pancreatic enzymes *in vitro* [13].

While milk proteins have been expressed in systems such as transgenic cows and *Aspergillus* [15], transgenic rice provides a more attractive vehicle for the production of recombinant human AAT for food applications. Rice is often one of the first foods introduced to infants because of its nutritional value and low allergenicity. Safety concerns about microbial expression systems (e.g. *Aspergillus*) limit the feasibility of using proteins from such sources as food components in formula [15]. In addition, the cost of producing and purifying proteins from these other systems is often prohibitive for food applications. Thus, expression of recombinant human milk proteins in rice may be a safe and economically viable possibility for supplementing infant formula with such proteins.

We have already expressed human lactoferrin and lysozyme in rice at high levels [8,16,17]. It is possible that the addition of recombinant human AAT together with these human milk proteins may protect them from proteolytic activity in the upper gut, and thus preserve their physiological function. However, testing of the biological activity of recombinant AAT is necessary before it can be considered as a possible candidate for supplementation. Thermal stability of the protein is important, since heat treatment is used to sterilize infant formulas. Stability at low pH is assessed to determine whether the protein can endure the mildly acidic environment of the infant stomach during digestion. An *in vitro* digestion model mimicking the gastrointestinal conditions of the infant can be used to predict the resistance of AAT to proteolytic degradation. We exposed recombinant AAT to such treatments in both phosphate buffered saline and infant formula, since the presence of formula may affect its stability during any of these conditions.

2. Materials and methods

2.1. Materials

Purification of functional recombinant human AAT has been reported previously [18]. Briefly, codon-optimized AAT gene was cloned into an expression cassette that contains the rice Ramy3D promoter, signal peptide, and terminator. Recombinant AAT expression was induced and secreted to the culture medium under the sugar starvation condition. Purification of rAAT was achieved through a scheme that consisted of an affinity column (Con A), anion exchange column, and a hydrophobic interaction column.

All reagents were obtained from Sigma Chemicals (St. Louis, MO). Human native AAT from serum, obtained from Athena Biochemicals (Athens, GA), was used since milk AAT is not commercially available. Rabbit anti-human AAT antiserum was obtained from Sigma, rabbit anti-human trypsin from Biodesign International (Saco, ME), rabbit anti-bovine serum albumin (BSA) from Dako Corporation (Carpinteria, CA), goat anti-AAT conjugated to horseradish peroxidase from ICN Biomedicals (Costa Mesa, CA), and donkey anti-rabbit IgG conjugated to horseradish peroxidase from Amersham Pharmacia Biotech (Piscataway, NJ).

The infant formula used in this study was Enfamil with Iron (Mead Johnson, Evansville, IL). This is a whey-based cow milk formula containing 14 g/L protein, 35 g/L fat, and 73 g/L carbohydrate.

2.2. Heat treatment

For the heat treatment, both native and recombinant human AAT were diluted in phosphate buffered saline (PBS) or infant formula (Enfamil with Iron) to a concentration of 0.1 g/L. Samples, 100 μl in capped, 10 x 75 mm glass tubes, were treated as follows: 60°C for 15 min, 72°C for 20 sec, 85°C for 3 min, and 137°C (temperature of oil bath) for 20 sec. The samples were allowed to cool to room temperature after heat treatment. For formula samples with bile extract added, 2.5 μl of 12% porcine bile extract (Sigma) were added, and then vortexed quickly, incubated at 37°C for 10 min, and vortexed again. All samples were diluted 1:10 in PBS and transferred to 1.5 ml tubes. Formula samples were centrifuged at 15,000 x g for 20 min to remove the insoluble fraction, and the supernatant was withdrawn after skimming off the fat. All samples were subsequently transferred to 1.5 ml tubes and analyzed.

2.3. pH treatment

Native and recombinant AAT were diluted in PBS or formula to 0.1 g/L. The sample volume was 1 ml, and the pH of each sample was adjusted drop-wise with 1 mol/L HCl. The range of pHs tested was from pH 2 to 8 for the samples in PBS and pH 2 to 7 for samples in formula. After a 1 h incubation at room temperature, the pH was restored to pH 7 with 1 mol/L $NaHCO_3$. Formula samples were centrifuged as above.

2.4. In vitro digestion

The procedure developed by Rudloff and Lonnerdal [19] was used after some modifications. Native and recombinant AAT were diluted in PBS or formula to 0.5 g/L. Hydrochloric acid (1 mol/L) was added to all samples to adjust the pH, then 2.5 μl of 2% porcine pepsin (Sigma) in 0.01 mol/L HCl (3,100 U/mg solid) were added and all samples were placed in a shaking incubator for 30 or 60 min at 37°C. The

pH was restored by drop-wise addition of 1 mol/L $NaHCO_3$, and 2.5 μl of 0.4% porcine pancreatin (Sigma) in 0.1 mol/L $NaHCO_3$ were added. Samples were incubated for 1 or 2 h at 37°C, and the reaction was halted by dilution 1:2 in sample buffer and boiling for 3 min. For samples subjected to pepsin digestion only, boiling was unnecessary since the pepsin was inactivated when the pH was raised above pH 6 with $NaHCO_3$ [20]. The enzyme: substrate ratio was approximately 1:20 for samples in buffer only and about 1:600 for samples in formula.

2.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

AAT samples were diluted 1:1 in sample buffer (1.25 mol/L Tris-HCl (pH 6.8), 10% SDS, 10% sucrose, 0.05% bromophenol blue), and applied (2 μg/well) to a 12% mini-gel (BioRad, Hercules, CA). Gels were run for 35 min at 200 V in Tris-glycine buffer (25 mmol/L Tris-HCl, pH 8.2, 0.19 mol/L glycine, 0.1% SDS). Gels were stained for 6 h in Coomassie Brilliant Blue, and destained with acetic acid: ethanol:water (10:25:65) for 12 h.

2.6. Western blot analysis

Following separation by SDS-PAGE, proteins were electroblotted onto a nitrocellulose membrane in Tris-glycine buffer (25 mmol/L Tris, 0.19 mol/L glycine, pH 8.2) at a constant current of 60 mA for 45 min at 4°C. The membrane was blocked overnight at 4°C in 4% bovine serum albumin or 8% fish gelatin (when BSA was used as substrate). Following blocking, the membrane was washed 3 times, 5 min per wash, in PBST (PBS, pH 7.4, 0.1% Tween-20) to reduce nonspecific binding of antiserum to the membrane. The membrane was incubated in a 1:5,000 dilution of rabbit anti-AAT, a 1:1,000 dilution of rabbit anti-human trypsin, or a 1:5,000 dilution of rabbit anti- bovine serum albumin for 1 h at room temperature, followed by 3 washes in PBST and incubation in a 1:5,000 dilution of donkey anti-rabbit IgG conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. The membrane was washed 3 times in PBST, and bound antibody was detected with a chemiluminescent system consisting of an HRP/hydrogen peroxide catalyzed reaction of luminol (Amersham).

2.7. ELISA

Samples and standards were assayed in triplicate. Standards for this assay ranged from 1.25-20 pg/L of AAT (Athena) diluted in PBST. Nunc Immuno-plate Maxisorp 96-well plates (Nunc, Denmark) were coated for 16 h at 4°C by a 1:10,000 dilution of rabbit anti-human AAT in 0.05 mol/L sodium bicarbonate, pH 9.6. The plates were washed 3 times with PBST (PBS, pH = 7.4, 0.05% Tween-20) and subsequently incubated with sample for 1 h at room temperature while rocking. The plates were washed again 3 times with PBST, followed by incubation with a 1:50,000 dilution of goat anti-human AAT conjugated to HRP for 1 h at room temperature. The plates were washed 3 times with PBST, and bound antibody was detected by the HRP/hydrogen peroxide catalyzed reaction of TMB. The reaction was stopped with 2 mol/L sulfuric acid, and the plates were read on a microtiter plate reader at 450 nm, using 620 nm as a reference filter.

2.8. Elastase activity assay

Samples and standards were assayed in triplicate. An adaptation of the elastase assay published by Travis and Johnson [21] was used. In 96-well microtiter plates, 60 μl samples diluted in Tris buffer, (0.2 mol/L Tris, pH 8.0) were added. In each well, 60 μl of elastase (0.01 g/L porcine elastase in Tris buffer) was also added. The plate was rocked for 5 min at room temperature to allow any available AAT to bind to the elastase. Another 120 μl of substrate solution (10 mol/L N-Succinyl-AAA-p-nitroanilide in DMSO diluted in Tris buffer to give 0.33 mol/L N-Succinyl-AAA-p-nitroanilide) was added, and the plate was rocked for 1-2 min at room temperature. The plate was immediately read on a microtiter plate at 405 nm. The plate was read again after 5 min, and the change in absorbance was calculated. AAT activity was determined using linear regression from a standard curve.

2.9. Trypsin activity assay

Native or recombinant AAT, 80 μl of 0.2 g/L AAT in PBS, pH 7.4, was incubated with 20 μl of porcine pancreatic trypsin, 0.2 g/L, for 5 min while rocking at room temperature. Bovine serum albumin (100 μl of 0.2 g/L solution) was added, and samples were incubated at 37°C for 1 h while shaking. Controls included BSA alone and BSA and trypsin without any AAT. Sample buffer was added at a 1:1 ratio, and the samples were boiled for 3 min. Samples were analyzed by SDS-PAGE and Western blots.

2.10. Statistical analysis

Data from the heat and pH treatments in Tables 1 and 2 were presented as mean ± standard deviation (SD) and statistical comparisons versus the control condition were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. Statistical comparisons of the pepsin digestion were analyzed by unpaired, 2-tailed Student t-test. GraphPad Prism software was used for all statistical analyses. [22]

3. Results

A comparison between native and recombinant AAT reveals some minor differences. Recombinant AAT is 2.1 times more immunoreactive (as assessed by ELISA, data

Table 1
Native and recombinant AAT stability against treatment at low pH.

Condition	Assay	AAT Type	pH treatment 2	3	4	5	6	7 control
PBS	ELISA	Native	93.5 ± 2.8	95.8 ± 1.5	96.9 ± 2.6	98.8 ± 5.8	94.7 ± 0.6	100 ± 6.3
		Rec	69.5 ± 13.3	78.9 ± 12.6	72.7 ± 14.7	68.4 ± 16.3	74.8 ± 19.2	100 ± 9.3
PBS	Elastase	Native	95.3 ± 1.8***	92.5 ± 1.5***	101.0 ± 2.0	101.6 ± 2.1	103.3 ± 2.4	100 ± 1.7
		Rec	70.0 ± 1.6***	67.6 ± 0.8***	71.1 ± 1.5***	78.6 ± 2.1	83.4 ± 2.3	100 ± 1.7
Formula	ELISA	Native	84.6 ± 6.4*	75.8 ± 10.3***	89.6 ± 9.1	81.8 ± 5.6***	85.3 ± 9.3*	100 ± 0.9
		Rec	78.4 ± 9.7***	89.6 ± 11.4	89.7 ± 4.9	91.7 ± 4.7	89.8 ± 9.0	100 ± 8.0

Values are expressed as percent detectable (mean ± SD) compared to pH 7 (control condition).
Asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) denote significantly different values from control, as determined by ANOVA followed by Dunnett's test.

not shown), when comparing equal concentrations as determined by protein assay. All results therefore are expressed as percent intact compared to control, so that the control is always designated as 100 percent intact. The recombinant form also has a slightly lower molecular mass, likely due to a lesser degree of glycosylation [23], but is able to bind elastase and trypsin.

Both native and recombinant AAT appear resistant to low pH conditions in both PBS and formula (Table 1). There were no differences between treatment groups and controls for pH 3 through 7, or between the native and recombinant AAT according to SDS-PAGE, Western blots, and trypsin assay. The elastase assay and ELISA data show that recombinant AAT is more affected by acidic conditions than the native form. In PBS, native AAT was more than 95% intact, while about 60-80% of the recombinant AAT activity was intact (Table 1). The pH treatment did not result in any statistically significant differences in the recombinant AAT because of the higher variability. While some of the data were significantly different from control conditions, native AAT was more than 95% intact under all conditions. Infant formula may have a stabilizing effect on the recombinant protein, since it was found to be as stable as the native form according to ELISA and the Western blot. (Table 1, Western blot not shown)

A significant amount of recombinant and native AAT survived the pepsin digestion (Fig. 1), and both forms were more resistant to degradation than human serum albumin (data not shown). Digestion with pepsin at pH 4 shows that 65% of recombinant AAT is detectable by ELISA after digestion, which is similar to the 67% of native AAT surviving. The trypsin assay shows that much of the inhibitory properties of both forms are still intact (Fig. 2), and the elastase assay reveals that 63% and 59% of the activity of native and recombinant AAT remains, respectively. These tests show that while a statistically significant amount of native and recombinant AAT is digested, a majority of AAT survives. In addition, the native and recombinant AAT respond similarly to pepsin treatment. When exposed to both pepsin and pancreatin in buffer, native AAT resisted degradation when the pH of the pepsin incubation was pH 4 or higher (Figs. 3a, b). Under this condition, the recombinant form was less resistant, although a large part remained after pepsin digestion at pH 5 and pancreatin digestion (Figs. 3a, b). At pH 4, more of the recombinant protein was degraded, either due to pepsin activity or pH instability. However, AAT activity could not be determined after digestion by pepsin and pancreatin because of the inactivation of pancreatin by boiling. In formula, both forms appeared to be equally resistant to degradation. While both native and recombinant AAT were still present after pepsin digestion at pH 5 followed by pancreatin digestion, bands at about 33 kD (casein) are faint or missing (Fig. 3c). It is possible that other proteins in formula are preferentially cleaved, reducing the amount of AAT being digested.

Table 2
Stability of native and recombinant AAT following pepsin digestion.

Condition	Assay	AAT Type	Treatment Control	Pepsin
PBS	ELISA	Native	100 ± 7.7	65.3 ± 6.9*
		Rec	100 ± 4.2	67.4 ± 2.7***
	Elastase	Native	100 ± 6.3	63.0 ± 4.3**
		Rec	100 ± 6.1	59.1 ± 5.7**

Values are expressed as percent detectable (mean ± SD) compared to undigested samples (control condition).
Asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) denote significantly different values from control, as determined by Student t-test (2-tailed, unpaired).

The thermal stability of native AAT exceeded that of the recombinant form in buffer, but the recombinant AAT retained significant stability under most conditions. When heated in buffer only, SDS-PAGE and Western blots show that the two forms of AAT have similar structural stability. For the native AAT, only the 85°C, 3 min condition is significantly different, and more than 90% is intact under all heat conditions. While the ELISA data (Table 3) show that the recombinant protein is less stable at the higher temperatures (85°C, 3 min and 137°C, 20 sec), the recombinant protein is similar to the native form under the other conditions. However, the functional stability of the recombinant protein may be affected. The trypsin assay (Fig. 4) shows that the recombinant protein lost functional ability at several of the heat conditions, whereas the native protein was func-

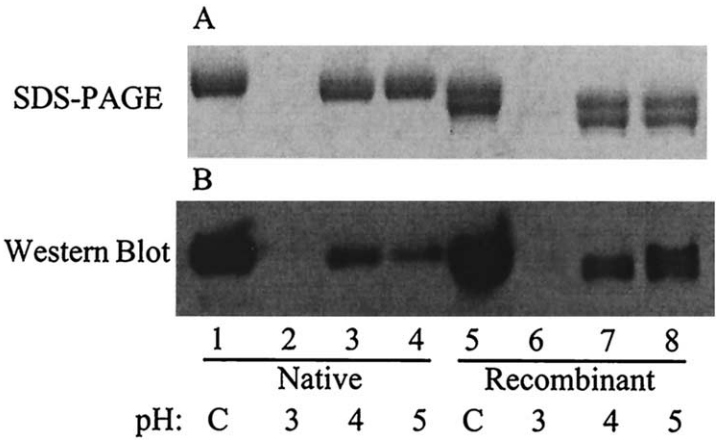

Fig. 1. Stability of AAT following pepsin digestion in PBS, assessed by SDS-PAGE and Western blot. The stated pH reflects the pH of the sample during the pepsin digestion. SDS-PAGE, reducing conditions, Coomassie-stained, and Western blot.

tional at all heat conditions except for at 62°C for 15 min. While the elastase-inhibiting properties of native AAT were intact after all heat treatments, only 29 ± 2 and 33 ± 1% (mean ± SD) of the recombinant protein's activity (data not shown) remained after 85°C, 3 min, and 137°C, 20 sec, respectively.

The heat treatments of native and recombinant AAT in formula affected the detection of the proteins, but the addition of bile extract following heat treatment restored antibody recognition of the recombinant form. While the Western blot data (Fig. 5a) show less detectable protein only at 85°C, 3 min for the native AAT and at 72°C, 20 sec and 137°C, 20 sec for the recombinant AAT, the ELISA data (Table 3) show less than 20% protein detected for both forms and for all heat conditions, and all were significantly lower when compared to the control conditions. When bile extract was added to the heated formula samples, the ELISA data for the recombinant form showed that more than 50% was still detectable after heat treatment (Table 3). While this proportion is still significantly lower than for the control conditions, it may still be enough to be functionally significant and feasible, and represents a significant improvement than without bile extract. The bile extract did affect detection of the native form by ELISA for most of the heat treatments. The Western blots corroborated the ELISA data and showed that the bile extract may dissociate the recombinant AAT from other formula proteins, but it is not effective for native AAT at the higher temperatures.

4. Discussion

Our results show that native and recombinant AAT can withstand acidic and digestive conditions as assessed by SDS-PAGE, Western blots, ELISA and elastase assay. Native AAT regains much of its structural and functional stability after treatment at acidic conditions followed by neutralization, whereas recombinant AAT shows some loss of activity at a wide pH range; this difference in stability between the native and recombinant form may be due to differences in glycosylation. When subjected to acidic conditions, Glaser et al. [24] showed that most of the trypsin-inhibiting properties of AAT are restored when reincubated at a basic or neutral pH. The pepsin incubation experiments suggest that a significant proportion of AAT can be functionally active after passing through the stomach and is

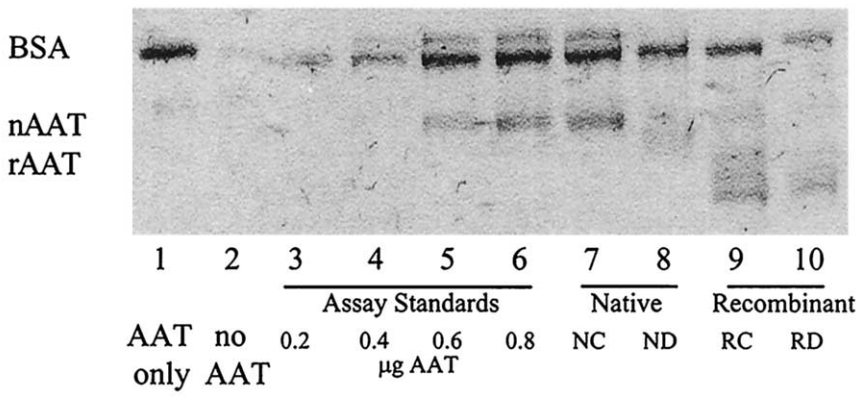

Fig. 2. AAT activity following pepsin digestion, assessed by trypsin assay.

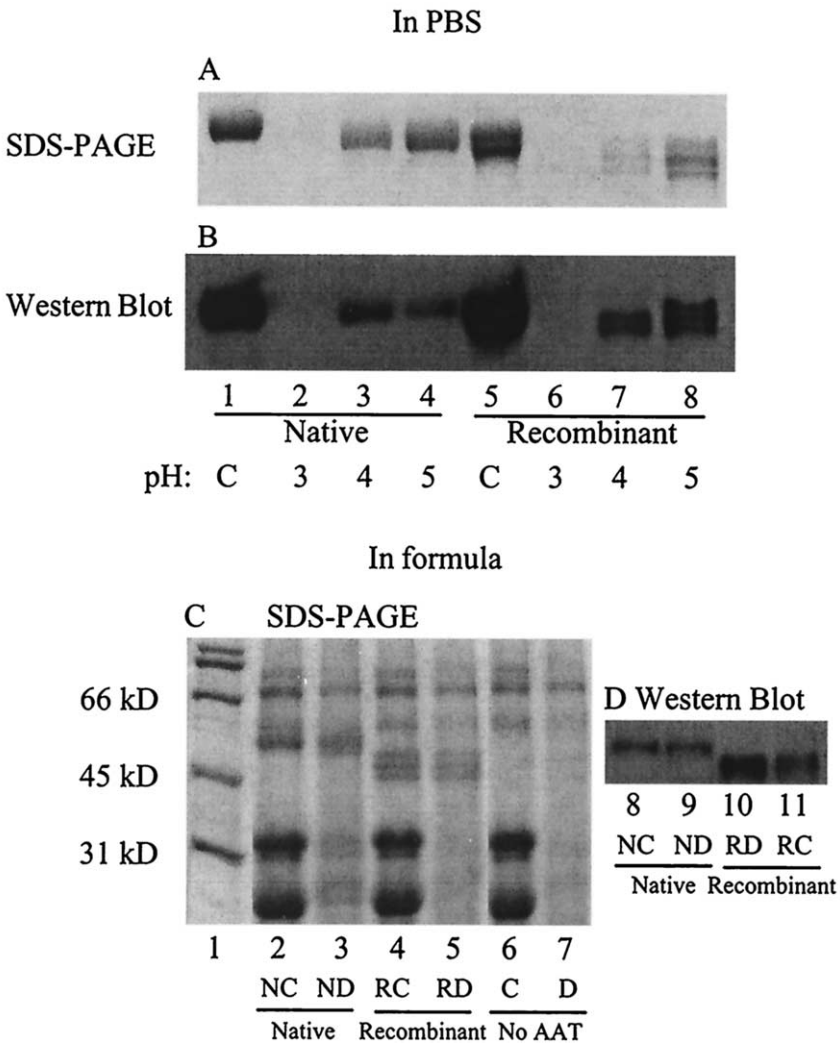

Fig. 3. Stability of AAT following pepsin and pancreatin digestion in PBS and milk formula, assessed by SDS-PAGE and Western blot. The stated pH reflects the pH of the sample during the pepsin digestion. Digestion in PBS: (A) SDS-PAGE, reducing conditions, Coomassie-stained, and (B) Western blot. Digestion in formula: (C) SDS-PAGE, reducing conditions, Coomassie-stained, and (D) Western blot. The pH during pepsin digestion of formula samples is 4.5.

capable of binding to trypsin in the duodenum. The *in vitro* digestion of AAT in formula shows that large amounts of casein (~33 kD) are digested, far more than the amount of AAT cleaved. Thus, formula proteins are likely to provide some protection of AAT by acting as substrates for proteases. Since recombinant AAT shows some loss after pH treatment, part of the loss during pepsin treatment may be due to the acidic conditions, and not just to pepsin activity. The conditions of the infant-modeled digestion, pH 5 during pepsin treatment, are not ideal for pepsin, which normally

Table 3
Stability of native and recombinant AAT following heat treatment.

Condition	Assay	AAT Type	Heat Treatment				
			Control	60°C, 15 min	72°C, 20 s	85°C, 3 min	137°C, 20 s
PBS	ELISA	Native	100 ± 10.4	92.1 ± 5.8	111.3 ± 4.5	82.7 ± 7.0***	91.4 ± 2.6
		Rec	100 ± 0.5	94.2 ± 5.3	102.1 ± 2.4	61.8 ± 4.3***	51.5 ± 12.8***
Formula	ELISA	Native	100 ± 3.4	3.5 ± 6.3***	7.9 ± 16.1	0 ± 11.2***	0 ± 3.3***
		Rec	100 ± 5.9	18.4 ± 2.6***	9.8 ± 0.8***	5.0 ± 1.2***	5.0 ± 0.8***
Formula w/ Bile	ELISA	Native	100 ± 2.3	25.5 ± 6.2***	71.3 ± 9.7***	0 ± 3.7***	4.9 ± 3.1***
		Rec	100 ± 1.3	66.3 ± 7.0***	71.6 ± 4.4***	53.7 ± 8.4***	55.3 ± 7.5***

Values are expressed as percent detectable (mean ± SD) compared to without heat treatment (control condition).
Asterisks (***$p<0.001$) denote significantly different values from control, as determined by ANOVA followed by Dunnett's test.

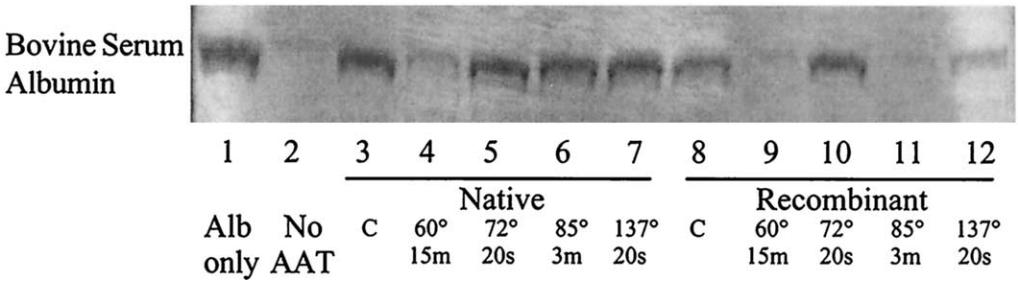

Fig. 4. AAT activity following thermal treatment, assessed by trypsin assay.

possesses full activity at pH 2 [20]. Under these conditions, human serum albumin is largely degraded, while human milk lactoferrin is mostly intact [16]. Lactoferrin has been shown to be relatively resistant against proteolytic enzymes [25]. AAT can survive similar *in vitro* digestion conditions in the presence of human milk [13], and AAT has been detected in human infant feces, which supports the notion that it is capable of surviving digestion *in vivo*, particularly during the first three months of the infant's life [10]. This evidence also supports the validity of the *in vitro* digestion system. It is therefore likely that AAT possesses enough resistance to acidic and digestive conditions to allow a significant amount to survive and affect the digestion process. *In vitro* digestion systems have been shown to correlate well with *in vivo* animal assays [26], but comparable human infant data are not available. Similar to other investigators [27], we used porcine pepsin and pancreatin in our assays, as they are commercially available; however, human AAT has been shown to bind to porcine enzymes [28-30].

AAT may aggregate with formula proteins when subjected to heat treatment. This physical aggregation may affect detection by antibodies, but may be less significant biologically. During digestion in the gut, bile salts emulsify lipids and also dissociate aggregated proteins. When we added bile extract to infant formula after exposure to heat treatment, more AAT was detected, particularly the recombinant form. The presence of detergent (SDS) during gel electrophoresis may have reduced the amount of aggregation and restored more sites for primary antibody binding, compared to ELISA. Since both forms of AAT display more resilience during the thermal treatment in buffer alone, formula appeared to reduce the amount of protein recognized by the primary antibody. It is thus possible that other milk proteins are aggregating with AAT and hiding its epitopes, making it less detectable. This suggests that heat treatment of recombinant AAT may only hide epitopes, but may not affect its functional or structural stability.

Recombinant AAT remained functionally intact after being exposed to low pH, *in vitro* digestion, and several types of heat treatment. It is therefore possible that recombinant AAT may be added to infant formula, can tolerate some processing conditions, and remain intact in the gastrointestinal tract of infants. More studies are required to determine the effects of infant stomach acidity and bile secretion on

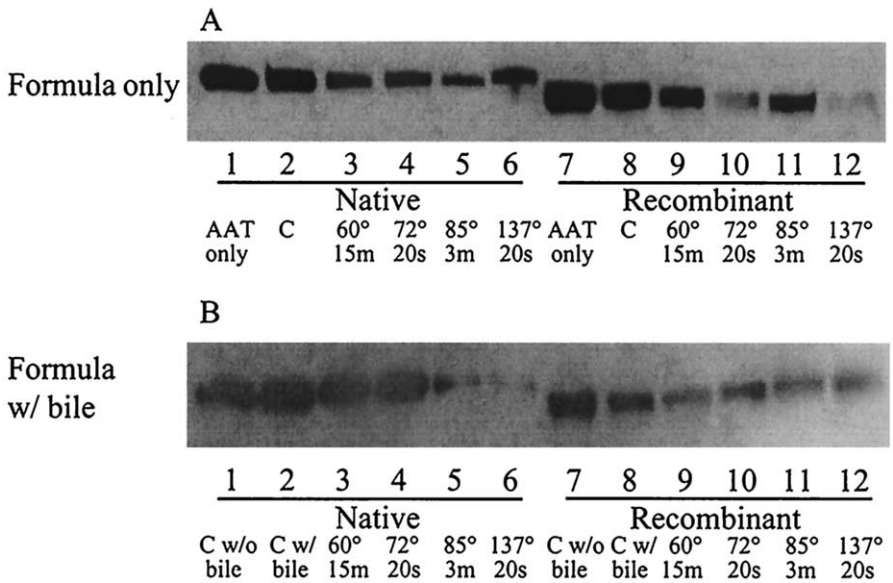

Fig. 5. Stability of AAT following thermal treatment of AAT in milk formula, with and without bile extract, assessed by Western blot. (A) No bile extract added after thermal treatment. (B) Bile extract added after thermal treatment.

recombinant AAT stability *in vivo*, possibly using an animal model. Thus, recombinant AAT may help protect other physiologically active proteins, such as lactoferrin and lysozyme, which also may be added in recombinant forms, in the gut of formula-fed infants. In conclusion, addition of recombinant AAT together with other recombinant proteins may enhance their bioactivity and make the formula more similar to human milk.

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