# A Novel Inhibitor of $\beta$-Glucuronidase: L-Aspartic Acid

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## ABSTRACT

Infants who consume casein hydrolysate formula have been shown to have lower neonatal jaundice levels than infants who consume routine formula or breast milk. Because casein hydrolysate has been shown to contain a $\beta$-glucuronidase inhibitor, one possible mechanism to explain this finding is blockage of the enterohepatic circulation of bilirubin by a component of the formula. The aim of this research was to identify the source of the $\beta$-glucuronidase inhibition in hydrolyzed casein. A $\beta$-glucuronidase inhibition assay and measurements of physical and kinetic parameters were used to analyze the components of hydrolyzed casein and infant formulas. Kinetic studies used purified $\beta$-glucuronidase. The L-aspartic acid in hydrolyzed casein accounts for the majority of the $\beta$-glucuronidase inhibition present. Kinetic studies indicate a competitive inhibition mechanism. L-Aspartic acid is a newly identified competitive inhibitor of $\beta$-glucuronidase. (***Pediatr Res** 50: 460–466, 2001*)

### Abbreviations

**$\beta$-glu,** $\beta$-glucuronidase
**D-gal,** D(+)galactose
**NAG,** *N*-acetyl-D-galactosamine
**NAN,** *N*-acetylneuraminic acid
**EAA,** essential amino acid solution
**NEAA,** nonessential amino acid solution
**MUG,** 4-methylumbelliferyl $\beta$-D-glucuronide
**MU,** 4-methylumbelliferone
**PG,** phenolphthalein mono-$\beta$-glucuronic acid
**NaAC,** sodium acetate/acetic acid buffer, pH 4.5
**SAB,** sodium acetate/acetic acid buffer, pH 5.0
**AMP,** 100 mM 2-amino-2-methyl-1-propanol buffer containing 0.2% (wt/vol) sodium lauryl sulfate at pH 11.0
**NRTU,** Nutramigen ready-to-use formula
**EHC,** enzymatically hydrolyzed casein
**NLFS,** Nutramigen lipid-free supernatant
**PLFS,** Phenyl-Free, lipid-free supernatant

In 1992, the new finding that all neonatal formulas are not equal in their effect on neonatal jaundice was first reported. Infants who consumed a casein hydrolysate formula had lower levels of jaundice than infants who received routine (whey or casein predominant) infant formulas (1). This finding was subsequently confirmed in an independent study (2). A potential mechanism has been proposed and a causative factor has been identified that might explain this phenomenon. The enterohepatic circulation of bilirubin could be interrupted by a $\beta$-glu inhibitor present in enzymatically hydrolyzed casein (3). Bilirubin, arising mainly from the degradation of heme, undergoes hepatic conjugation with glucuronic acid and excretion as bilirubin glucuronides via bile into the intestine. Intestinal $\beta$-glu can cleave the glucuronide linkage to produce unconjugated bilirubin, which can be absorbed by the intestine back into the blood circulation (4). Inhibition of $\beta$-glu can block this enterohepatic circulation of bilirubin, thus facilitating fecal excretion and lower serum bilirubin concentration (5). Inhibitors of $\beta$-glu have also been suggested to have anticarcinogenic properties because they will increase clearance of glucuronidated carcinogens (6).

Because neonatal jaundice is the most common cause for hospital readmission of neonates (7), any potential new therapeutic considerations are of interest. Identification of any $\beta$-glu inhibitor that is currently acknowledged to be a safe dietary element would be of considerable appeal. In this paper, data are presented which demonstrate that L-aspartic acid (L-asp) is one component of hydrolyzed casein infant formula that is inhibitory to $\beta$-glu. L-asp has not previously been identified as a $\beta$-glu inhibitor.

## METHODS

***Materials.*** The following supplies and chemicals were obtained from Sigma Chemical (St. Louis, MO, U.S.A.): D-gal, NAG, NAN, amicase, EAA [containing L-arginine (Arg), L-cystine (Cys), L-histidine (His), L-isoleucine (Ile), L-lysine (Lys), L-methionine (Met), L-phenylalanine (Phe), L-threonine (Thr), L-tryptophan (Trp), L-tyrosine (Tyr), and L-valine (Val)],

NEAA [containing L-alanine (Ala), L-asparagine (Asn), L-aspartic acid (L-asp), L-glutamic acid (L-glu), glycine (Gly), L-proline (Pro), and L-serine (Ser)], D-aspartic acid (D-asp), D-glutamic acid (D-glu), aspartic acid dimer ([asp]$_2$), aspartic acid trimer ([asp]$_3$), aspartic acid tetramer ([asp]$_4$), aspartic acid pentamer ([asp]$_5$), bovine liver β-glu G-4882 (glucurase), glycerol, BSA fraction V, EDTA, sodium acetate, sodium phosphate, MUG, MU, citric acid monohydrate, oxalic acid dihydrate, L-malic acid (L-mal), D-malic acid (D-mal), L-tartaric acid (L-tar), D-tartaric acid (D-tar), dibasic sodium phosphate heptahydrate, 30 mM PG solution at pH 4.5, 0.2 M NaAC at pH 4.5, 0.2 M SAB at pH 5.0, 100 mM AMP at pH 11.0, 1 mg/mL phenolphthalein standard solution in ethanol, and dialysis tubing 250-7U (retains proteins with molecular weight >12,000 D).

The following infant formulas and components were obtained from Mead Johnson Nutritionals (Evansville, IN, U.S.A.): NRTU, Nutramigen powder formula, Phenyl-Free (phenylalanine-free powder), and EHC. Nutramigen formula from powder, EHC suspension (2.2% wt/vol), and NLFS solutions were prepared as described (3). PLFS was prepared similarly to NLFS. Phenyl-free formula was prepared from powder by using the standard recommendation: 101 g powder to 14 fl oz water.

***Dialysis and freeze/thaw/boiling experiments.*** EHC suspension [16.3% (wt/vol), 15 mL] was dialyzed 24 h against 6 L of deionized water. The original EHC and the dialyzed EHC solutions were diluted 5X with NaAC buffer and tested for β-glu inhibition by using the human milk β-glu inhibition assay (see below). A 2.2% (wt/vol) EHC suspension was prepared in NaAC buffer. The EHC suspension was diluted 5X with NaAC buffer and divided into two aliquots. One aliquot was maintained at room temperature and immediately assayed using the human milk β-glu inhibition assay (see below). The second aliquot was frozen at −20°C overnight, thawed, heated to 100°C for 10 min, cooled to room temperature, and tested for β-glu inhibition. The final pH was 4.5 for both aliquots. The final assay mixture contained human milk diluted 25-fold and an EHC concentration of 0.176% (wt/vol).

***β-Glu inhibition assay (β-glu source: human milk).*** Human breast milk samples that had been collected independently of this research were obtained from stored unused residuals at the newborn nursery of Meriter Hospital (Madison, WI, U.S.A.) and stored at −20°C. These samples were combined, and aliquots were used for the studies described. The milk was thawed and diluted 10–20X with NaAC buffer just before use. The β-glu activity was measured fluorometrically (Perkin Elmer LS-5B fluorescence spectrophotometer; excitation, 360 nm; emission, 445 nm) using MUG as substrate at 37°C for 1 h (or other times as indicated) using NaAC buffer as previously described (3). The β-glu activity in the presence or absence (controls) of potential inhibitory substances was calculated as pmol of 4-MU formed per h/mL undiluted breast milk. The extent of inhibition was calculated based on the velocity of the controls.

***β-Glu inhibition as a function of L-asp concentration.*** β-Glu activity was measured as a function of L-asp concentration. The concentration of L-asp was determined either with a Beckman 6300 automatic amino acid analyzer (8) for the EHC, NLFS, and PLFS or gravimetrically (±0.00001 g) for the L-asp solutions. The following samples were assayed in NaAC buffer (as described above) at the final indicated concentrations of L-asp (μM): control (0), NLFS (184), EHC (208), L-asp (2000), L-asp + EHC (2208), L-asp (4000), L-asp + EHC (4208), PLFS (4482), L-asp (6000), and L-asp + EHC (6208). The final concentration of MUG was 640 μM. The milk was diluted 50X, and original EHC, NLFS, and PLFS suspensions were diluted 12.5-fold in the final assay mixture.

***β-Glu inhibition and kinetic assay (β-glu source: bovine liver, glucurase).*** Glucurase liquid was diluted with SAB buffer to form diluted stock solutions ranging from 0.5 to 10.0 U/mL (1 U will liberate 1.0 μg phenolphthalein from phenolphthalein glucuronide per hour at 37°C at pH 5.0). Stock solutions of MUG ranging from 13 to 10,000 μM were prepared in SAB buffer. Stock solutions of L-asp were prepared in SAB buffer at 2.5X the final assay concentration. The assay was run as described above with appropriate modifications. Assays included 50 μL of a stock L-asp solution or buffer (0 μM L-asp). Triplicate tests and blanks were prepared by adding 25–100 μL of the diluted glucurase. The assay was started by adding 25–100 μL of one of the stock MUG solutions to the tests. The final volume of the assay was always 125 μL. The velocity was calculated as pmol of 4-MU formed per h/U of bovine liver β-glu. Kinetic analysis was performed as described below.

***β-Glu inhibition assay using various pH conditions (β-glu source: human milk or glucurase).*** Either pooled human breast milk (see above) or glucurase (5000 U/mL) was used as the β-glu source. Distilled water was used to dilute human milk 5X or glucurase to form a diluted stock solution of 10.0 U/mL. A 1.6-mM stock solution of MUG was prepared in distilled water at 10X the final assay concentration. A 25 mM stock solution of L-asp was prepared in distilled water at 5X the final assay concentration. Citrate-phosphate buffers ranging in pH from 3.5 to 7.8 were prepared (9) and confirmed by pH meter. Triplicate tests and blanks were prepared by adding 150 μL of the appropriate citrate-phosphate buffer, 25 μL of diluted human milk or glucurase, and 50 μL of either the stock L-asp solution (treatment, 5000 μM L-asp) or distilled water (control, 0 μM L-asp). The assay was started by adding 25 μL of the stock MUG solutions to the tests. The final volume of the assay mixture was 250 μL. The remainder of the assay was run as described above. The final assay enzyme concentration was a 50-fold dilution of the pooled human milk. The final glucurase concentration was 1 U/mL. The final concentrations of MUG and L-asp was 160 and 5000 μM. The velocity was calculated as the pmol of 4-MU formed per h/mL of undiluted human breast milk or per unit glucurase.

***β-Glu inhibition assay using phenolphthalein glucuronide as a substrate (β-glu source: bovine liver, glucurase).*** A method based on the Sigma Chemical Co. Diagnostics Procedure No. 325 for the colorimetric (550 nm) determination of β-glu was used to measure glucurase activity in the presence or absence of L-asp. Glucurase liquid (5000 U/mL) was diluted with NaAC buffer to provide a 5X stock solution containing 200 U of enzyme per mL. Serial dilutions of the 30 mM PG

were performed using NaAC buffer to prepare 5X stock PG solutions ranging from 187.5 to 30,000 $\mu$M. A 50 mM stock solution of L-asp was prepared in NaAC buffer at 5X the final assay concentration. Triplicate reagent blanks, controls, and treatments were prepared by adding *1*) 200 $\mu$L of NaAC buffer to all tubes, *2*) 100 $\mu$L of the appropriate PG stock solution to all tubes, *3*) 100 $\mu$L of either the stock L-asp solution (treatment and reagent blank) or 100 $\mu$L of 200 mM NaAC (control). The assay was started by adding 100 $\mu$L of diluted enzyme stock solution to the control and treatment tubes and 100 $\mu$L of NaAC buffer to the reagent blank tubes. The final volume of the assay mixture was 500 $\mu$L. All tubes were incubated at 56°C for 1 h. The reaction was quenched with 3 mL of AMP buffer, and the absorbance at 550 nm was determined using a Turner model 690 spectrophotometer. Final concentrations were glucarase, 40 U/mL; L-asp, 10 mM; and PG, ranging from 37.5 to 6000 $\mu$M. The amount of phenolphthalein released by the $\beta$-glu enzyme was determined by using a linear standard calibration curve (dilutions of the Sigma Chemical Co. standard 1 mg/mL phenolphthalein solution) and the Mathplot software (version 1.1, Physics Academic Software, Raleigh, NC, U.S.A.).

***β-Glu polycarboxylic acid inhibition assay (β-glu source: human milk or glucarase).*** Either pooled human breast milk (see above) or glucarase was used as the $\beta$-glu source. The glucarase was diluted with SAB buffer to a concentration of 10 U/mL and used as a 10X enzyme stock solution. A 1.6 mM stock solution of MUG was prepared in SAB buffer at 10X the final assay concentration. Stock carboxylic acid solutions (25 mM) were prepared in SAB buffer at 5X the final assay concentration. Polycarboxylic acids were studied because they are structurally related to aspartic acid and because some are known inhibitors of $\beta$-glu. Triplicate tests and blanks were prepared by adding 150 $\mu$L of the SAB buffer, 25 $\mu$L of human breast milk or glucarase, and 50 $\mu$L of the stock carboxylic acid solution or distilled water (control). The assay was started by adding 25 $\mu$L of the stock MUG solution to the tests. The final volume of the assay mixture was 250 $\mu$L. The remainder of the assay was run as described above. The original pooled breast milk was diluted 10-fold in the final assay. The final assay concentration of the glucarase was 1 U/mL. The final assay concentrations were 160 $\mu$M for the substrate (MUG) and 5000 $\mu$M for the carboxylic acids. The amount of MU formed was determined using the appropriate standard curve. The velocity was calculated as the pmol of 4-MU formed per h/mL of undiluted human breast milk or per unit of glucarase.

***Kinetic analysis methods.*** ROSFIT nonlinear regression software (10) was used to calculate kinetic parameters that depend upon the particular assumptions of each model. The following symbols were used: $V_{max}$, the enzyme maximum velocity; $K_m$, the Michaelis substrate affinity constant; and $K_i$, the dissociation constant for the enzyme-inhibitor complex. The mixed procedure (SAS Institute, Cary, NC, U.S.A.) was used to analyze the covariance of the slope and intercept parameter estimates used in the Lineweaver-Burk method of calculation of $V_{max}$ and $K_m$. Regression analysis using Statistica statistics software (Statsoft, Tulsa, OK, U.S.A.) was used when using the Dixon, Lineweaver-Burke, Hanes-Woolf, Eadie-Scatchard, Woolf-Augustine-Hofstee, and direct linear plot graphic methods (11) for determining $V_{max}$, $K_m$, and $K_i$.

## RESULTS

The $\beta$-glu assay used human breast milk as $\beta$-glu source and was linear through 60 min. Thus, for all subsequent assays, a standard assay time of 60 min was used. Dialysis data showed that $\beta$-glu inhibition decreases when EHC is dialyzed across a membrane with a 12,000-D cutoff [predialysis, 22.9% of control; postdialysis, 77.0% of control ($p < 10^{-7}$). The effect of freeze, thaw, and boiling on the ability of EHC to inhibit $\beta$-glu was examined. There was little change in the degree to which EHC inhibited $\beta$-glu before or after freeze, thaw, and boiling of the EHC (pre, 69.9% of control; post, 72.5% of control).

To determine whether $\beta$-glu inhibition was still present after complete hydrolysis of casein, amicase was assayed for $\beta$-glu inhibition. Amicase, produced by the acid hydrolysis of casein, is a mixture of free amino acids with virtually no unhydrolyzed peptides. The amicase concentration used was equivalent to the concentration of EHC in Nutramigen reconstituted from powder (2.2%). Amicase was found to significantly inhibit $\beta$-glu activity (71.3% of control, $p < 10^{-10}$).

Casein is a glycoprotein in which the protein component is conjugated to a polysaccharide. This polysaccharide makes up approximately 5% of the weight of the total glycoprotein and is principally composed of D-gal, NAG, and NAN (12). Experiments showed that these three carbohydrates, ranging in concentration from 0.1 to 10 mM, demonstrated no significant inhibitory effect on human milk $\beta$-glu.

Table 1 shows the effects of two different amino acid mixtures on $\beta$-glu activity. The EAA mixture is significantly stimulatory to $\beta$-glu. The NEAA mixture is significantly inhibitory to $\beta$-glu. Table 2 shows the effect of the specific amino

**Table 1.** *Effects of amino acid mixtures on β-glu activity*

| Parameter | C | NRTU | EAA | NEAA |
|---|---|---|---|---|
| % of Control | 100 | 35.3 | 110 | 68.3 |
| Velocity (nmol·mL$^{-1}$·h$^{-1}$) ($\bar{X} \pm$ SD) | 138 ± 4.7 | 48.8 ± 0.4 | 152 ± 1.7 | 94.4 ± 3.2 |
| $p^*$ | — | $<10^{-12}$ | $<10^{-4}$ | $<10^{-8}$ |

\* Unpaired *t* test *vs* control (C).

Inhibition of $\beta$-glu by NRTU, EAA, and NEAA was assayed ($n = 6$). NRTU was diluted 5X with NaAC buffer. EAA was diluted 5X with the NaAC buffer, resulting in final assay concentrations of 240 $\mu$M Arg, 400 $\mu$M Cys, 800 $\mu$M His, 1600 $\mu$M Ile, 1600 $\mu$M Leu, 1592 $\mu$M Lys, 408 $\mu$M Met, 800 $\mu$M Phe, 1600 $\mu$M Thr, 200 $\mu$M Trp, 792 $\mu$M Tyr, and 1600 $\mu$M Val. NEAA was diluted 10X with NaAC buffer, resulting in final assay concentrations of 400 $\mu$M Ala, 400 $\mu$M Asn, 400 $\mu$M L-asp, 400 $\mu$M L-glu, 400 $\mu$M Gly, 400 $\mu$M Pro, and 400 $\mu$M Ser. The human milk was diluted 25-fold in final assay mixture.

**Table 2.** *Effect of specific amino acids on β-glu activity*

| Parameter | Control | Ala | Asn | L-asp | L-glu | Gly | Pro | Ser |
|---|---|---|---|---|---|---|---|---|
| % of Control | 100 | 99.5 | 97.8 | 73.7 | 97.9 | 101.7 | 99.3 | 99.3 |
| Velocity $(nmol \cdot mL^{-1} \cdot h^{-1})$ | | | | | | | | |
| $\overline{X}$ | 135 | 134 | 132 | 99.3 | 132 | 137 | 134 | 134 |
| (SD) | (4.1) | (3.0) | (2.2) | (0.7) | (0.7) | (2.1) | (1.8) | (1.9) |
| $p*$ | — | 0.83 | 0.29 | $<10^{-5}$ | 0.29 | 0.40 | 0.73 | 0.71 |

* Unpaired *t* test *vs* control.

Inhibition of β-glu by specific individual amino acids was assayed (*n* = 3). Stock solutions (1 mM) of each amino acid were prepared in NaAC buffer and final concentration was 400 $\mu$M. Human milk was diluted 25-fold in the final assay mixture.

acid components in the NEAA mixture on the inhibition of β-glu. The concentrations tested were the same as those of Table 1. Of these seven individual amino acids, only L-asp was significantly inhibitory to β-glu. The inhibition associated with L-asp was comparable to that seen in the NEAA mixture (73.7 *versus* 68.3% of control, respectively).

The effects of L- *versus* D-asp on β-glu activity were examined at concentrations ranging from 10 $\mu$M to 10 mM. L-asp showed significant β-glu inhibitory activity at all concentrations greater than 10 $\mu$M. The inhibition by L-asp showed a dose response with the maximal inhibition (8.4% of control) at 10 mM. D-asp only demonstrated significant inhibition at the highest concentration tested, 10 mM (86.2% of control). The inhibition by 10 mM D-asp was approximately equal to that of 100 $\mu$M L-asp. Thus, L-asp is approximately 100 times more potent than D-asp in the inhibition of β-glu.

Table 3 presents the effects of aspartic acid and its polymers on β-glu activity. L-asp, 1000 $\mu$M, demonstrates the maximal inhibition (49.2% of control). D-asp, 1000 $\mu$M, shows no inhibition. Aspartic acid polymers ranging in size from 2 to 5 repeating asp units are less inhibitory than L-asp. As the polymer chain length increases, the β-glu inhibitory effect decreases until a chain length of 3. As polymer chain length continues to increase ([asp]$_4$ and [asp]$_5$), there is a stepwise increase in inhibition. Because L-asp is inhibitory to β-glu, the effect of various concentrations of L-asp (in several different products or stock solutions) on β-glu activity was examined. These results are shown in Figure 1 as a Dixon plot. The regression line for the plot is positive with a regression coefficient near unity ($R = 0.991$, $p < 0.001$), raising the possibility that the L-asp concentration in these various samples is what is responsible for the inhibition of β-glu.

To determine more about the kinetics of β-glu inhibition by L-asp, experiments were performed using a preparation of purified bovine liver β-glu (glucurase). Initial experiments

indicated that at a fixed concentration of substrate (800 $\mu$M MUG), activity was linear up to a glucurase concentration of 3.2 U/mL. In the next experiments, two different glucurase concentrations were used, 1 and 2 U/mL, both within this linear region, and the substrate (MUG) concentration was varied from 13 to 10,000 $\mu$M. These data, when plotted in double reciprocal fashion, revealed no significant difference in the slope (8.98 *versus* 9.08) or the *y* intercept (165.9 *versus* 166.8) between the 1 and 2 U/mL concentrations, respectively. Subsequent kinetic studies used a final enzyme assay concentration of 1 U/mL.

Various graphic and computational methods were used to determine the model and kinetic constants for the inhibition of glucurase by L-asp. Analysis of the graphs suggested competitive inhibition (*e.g.* see Fig. 2). $V_{max}$ ranged from 9,815 to 10,000 $\mu$M, $K_m$ ranged from 142–150 $\mu$M, and $K_i$ ranged from 5000 to 5100 $\mu$M. ROSFIT computational analysis also showed that a competitive inhibition model was the best fit for the data (lowest s-squared), with $V_{max}$ of 10,052 $\mu$M, $K_m$ of 147 $\mu$M, and $K_i$ of 4387 $\mu$M.

Figure 3 indicates the effect of pH on the inhibition of β-glu. The concentration of L-asp was 5000 $\mu$M, close to the $K_i$, and the concentration of MUG was 160 $\mu$M, close to the $K_m$. Panel A (glucurase) shows a maximal inhibition of 64.6% of control at pH 5.0. Panel B (human milk β-glu) shows a maximal inhibition of 37.9% at pH 5.0.

To further assess the inhibition of β-glu by L-asp, another common substrate, PG, was used. Figure 4 compares L-asp inhibition of β-glu using either MUG or PG. Panel A shows the classic Michaelis-Menten curve with velocity asymptotically approaching $V_{max}$ as MUG concentration increases. At high MUG concentration, velocity is essentially independent of substrate concentration. Panel B shows that at high PG concentration, velocity decreases, consistent with substrate inhibition. However, with both substrates, L-asp demonstrates inhi-

**Table 3.** *Effect of aspartic acid and aspartic acid polymers on β-glu activity*

| Parameter | C | D-asp | L-asp | [asp]$_2$ | [asp]$_3$ | [asp]$_4$ | [asp]$_5$ |
|---|---|---|---|---|---|---|---|
| % | 100 | 100 | 49.2 | 58.7 | 87.3 | 75.9 | 64.6 |
| Velocity $(nmol \cdot mL^{-1} \cdot h^{-1})$ | | | | | | | |
| $\overline{X}$ | 136 | 137 | 67.0 | 80.0 | 119 | 103 | 88.1 |
| (SD) | (4.4) | (7.8) | (4.3) | (0.7) | (4.1) | (2.1) | (2.3) |
| $p*$ | — | 0.94 | $<10^{-7}$ | $<10^{-6}$ | $<10^{-3}$ | $<10^{-5}$ | $<10^{-6}$ |

* Unpaired *t* test *vs* control (C, *n* = 6).

Inhibition of β-glu by L-asp, D-asp, and aspartic acid polymers was assayed (*n* = 3). Stock solutions (2.5 mM) of L-asp, D-asp, [asp]$_2$, [asp]$_3$, [asp]$_4$, and [asp]$_5$ were prepared in NaAC buffer; final concentrations were 1 mM.
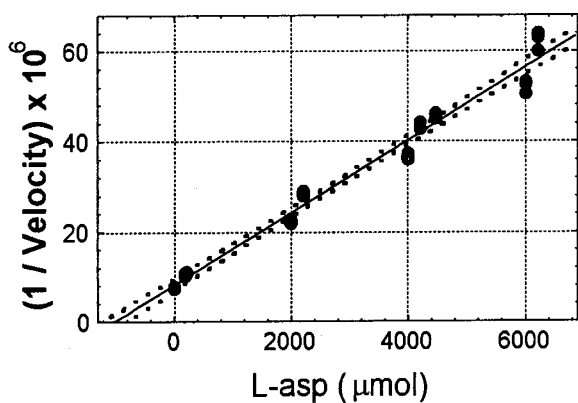
**Figure 1.** L-asp concentration *vs* the reciprocal velocity of human milk β-glu. β-Glu activity is presented as a function of L-asp concentration (see "Methods"). *Solid line* indicates regression line ($R = 0.991$, $p < 0.001$); *dotted lines*, 95% confidence limits.
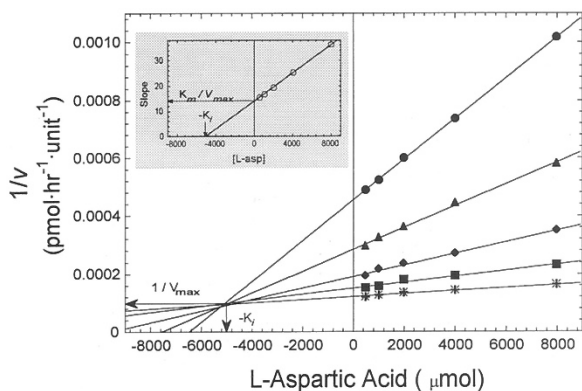


**Figure 2.** Kinetic plots for the inhibition of bovine liver β-glu (glucurase) by L-asp. The *large graph* is a Dixon plot of reciprocal velocity as a function of L-asp concentration at various concentrations of MUG. The final enzyme concentration was 1 U/mL. MUG concentrations: 640 $\mu$M (*solid star*), 320 $\mu$M (*solid square*), 160 $\mu$M (*solid diamond*), 80 $\mu$M (*solid triangle*), and 40 $\mu$M (*solid circle*). The *inset* depicts the slopes ($\times 10^3$) of the Lineweaver-Burke double reciprocal plot (not shown) for the same data (*open circles*).
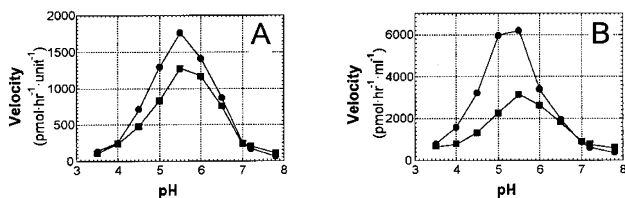


**Figure 3.** Effect of pH on the L-asp inhibition of β-glu from bovine liver (*A*) or human milk (*B*). Controls (*circles*), L-asp 5 mM (*squares*).

bition (dashed lines in panels A and B). The shaded insets present double reciprocal plots demonstrating that for PG, there can be both competitive inhibition and substrate inhibition (increasing $1/v$ as $1/S$ approaches zero) depending on the substrate concentration.

Figure 5 compares the β-glu inhibition by carboxylic acids, which are structurally related to L-asp. Both glucurase and human milk β-glu were examined. At equal concentrations (5000 $\mu$M), only L-mal was more inhibitory to the human milk β-glu than L-asp ($p < 0.001$, *t* test).
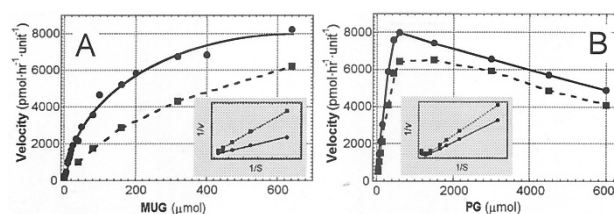


**Figure 4.** Velocity *vs* substrate concentration using two different substrates, MUG (*A*) or PG (*B*). β-Glu assay using glucurase [control, *solid lines*; added L-asp, *dashed lines*; 8 mM in (*A*), 10 mM in (*B*). See "Methods"]. *Shaded insets* show double reciprocal plots.
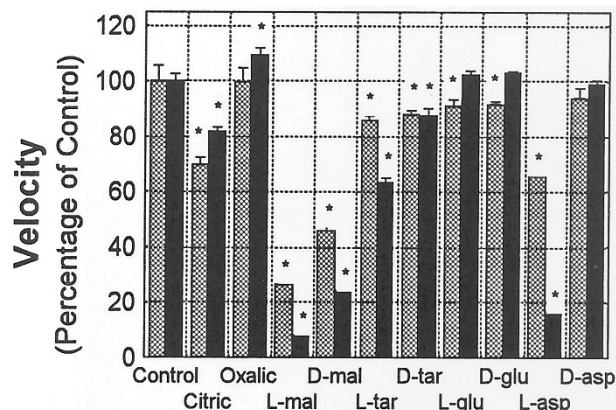


**Figure 5.** Inhibition of β-glu by polycarboxylic acids. Glucurase (*cross-hatch*) or human milk (*solid*). *Error bars*, 1 SD; *asterisk*, $p < 0.05$ *vs* controls (*t* test). Control: glucurase (1 U/mL), $3034 \pm 176$ pmol·h$^{-1}$·U$^{-1}$; human milk (10-fold dilution), $26227 \pm 688$ pmol·h$^{-1}$·mL$^{-1}$.

## DISCUSSION

β-Glu is a common and much studied enzyme that cleaves almost any aglycone in a β linkage to glucuronic acid (13). Enzymatically active β-glu is a tetrameric glycoprotein present in microsomes and lysosomes of many different organs and body fluids (13), including neonatal intestinal contents and human milk (14). β-Glu has long been thought to be important in the enterohepatic circulation of such substrates as bilirubin that undergo hepatic glucuronidation, excretion in the bile, and elimination in the feces (15). Intestinal β-glu is detectable in the human fetus as early as 8–12 wk gestation (16) and is important for clearance of bilirubin from the fetus via the placenta.

Much has been written about specific inhibitors of β-glu. Perhaps the best known of these is an analogue of glucuronic acid, the powerful competitive inhibitor glucaro-1,4-lactone (saccharolactone) (13). Gourley *et al.* (3) have shown that 10 $\mu$M saccharolactone will inhibit human milk β-glu by over 90%. Many other inhibitors of β-glu have been described and include an analog of nojirimycin (17), sumarin, alginic acid and particular polycarboxylic acids (13, 18), sodium heparin (19), and organic peroxides (20). The general disadvantage of these inhibitors is that they are not compounds which, at significant concentrations, are safe dietary ingredients.

In the present study, L-asp is reported to be a newly identified inhibitor of β-glu. The data presented support competitive inhibition as the mechanism. Aspartic acid (molecular weight, 133.1) is a nonessential amino acid present in human

milk and infant formula as both the free amino acid and in protein. The total amount of aspartic acid in mature human milk has been reported as 89 mg/dL (21), though free aspartic acid levels are much lower, ranging from 0.16 to 0.7 mg/dL and varying with gestational age and milk maturity (22). Total L-asp in cow milk is reported to be 166 mg/dL (23). The total L-asp concentration of the routine infant formula Enfamil is 132 mg/dL, and the L-asp concentration of casein hydrolysate formula Nutramigen is 152 mg/dL (personal correspondence, Mead Johnson). The majority of L-asp found in Enfamil is in intact protein, whereas, in Nutramigen, approximately 20% is the free amino acid and the remainder is in peptide fragments. It would be anticipated that the free amino acid would be more readily available to bind to the catalytic site and cause inhibition than L-asp which is constrained by being incorporated within protein. Phenyl-Free, a specialty formula lacking phenylalanine, developed for infants with phenylketonuria, has 920 mg L-asp/dL (personal correspondence, Mead Johnson) of which the current study found 742.4 mg/dL as free L-asp. If L-asp does block the enterohepatic circulation of bilirubin, one would expect to see serum bilirubin concentrations that are inversely proportional to the dietary intake of L-asp. Thus, among the three dietary groups for which such data exist, serum bilirubin concentrations would be expected to be lowest in Nutramigen-fed neonates, intermediate in Enfamil-fed neonates, and highest in breast-fed infants, just as has been reported in two independent studies (1, 2).

The finding that L-asp is an inhibitor of $\beta$-glu fits well with previous observations that other dicarboxylic acids are inhibitors of $\beta$-glu. The dietary safety of aspartate and glutamate has been examined extensively. There is no significant danger to healthy humans from the ingestion of these compounds under anything resembling a reasonable intake (24–26). The safety of EHC-based formulas and Phenyl-Free formula has been established, and such products have been on the market for many years. The American Academy of Pediatrics suggests that breast-fed infants admitted with high bilirubin levels and dehydration would best receive supplemental fluid as "...a milk-based formula, because it inhibits the enterohepatic circulation of bilirubin and helps lower the serum bilirubin level (27)." Although some breast-feeding advocates are against any formula supplementation of breast-fed infants, we speculate that use of small amounts of L-asp alone might be sufficient to inhibit the enterohepatic circulation and achieve lower serum bilirubin levels without use of artificial cow milk-based formula. More than 30 y ago, aspartic acid, a uridine precursor, was regarded as a possible therapy for neonatal jaundice in the belief that aspartic acid administration would increase uridine diphosphoglucuronic acid concentration and resultant hepatic bilirubin conjugation. In 1966, aspartic acid, 200 mg/d, given to 16 full-term newborn Japanese infants (diet, formula; personal communication, I. Matsuda) starting at the eighth hour of life and continued until the fourth day of life was associated with significantly lower serum bilirubin levels from d 2 to 5 after birth (28). However, a subsequent Scottish double-blind study of 12 preterm formula-fed infants found no effect of aspartic acid on serum bilirubin concentrations when the aspartic acid was given at a maximal dose of 200 mg/d for each

of the first 6 d of life (29). This negative study marked the end of investigations regarding the effects of aspartic acid on neonatal jaundice. Subsequently, two independent studies that included a total of 34 infants fed Nutramigen demonstrated significantly lower levels of jaundice in the Nutramigen group than in infants fed Enfamil or breast milk (1, 2). We suggest that these findings are not related to any effect of aspartic acid on uridine diphosphoglucuronic acid but rather to a differential effect on the enterohepatic circulation of bilirubin due to the inhibition of endogenous (meconium) and exogenous (breast milk) sources of intestinal $\beta$-glu. The normal pH of various portions of the gastrointestinal tract is in the range (pH 3.5–7.0) in which the L-asp shows significant *in vitro* inhibition. Therefore, we would expect that L-asp would exhibit a significant inhibitory effect on the $\beta$-glu found in the intestine resulting from human milk consumption and/or endogenous enzyme sources (bile, sloughed cells, and bacteria). At other portions of the gastrointestinal tract outside this pH range, we would expect lowered inhibitory effect of L-asp. We hypothesize that supplementation of breast-fed infants with small volumes of L-asp or other dietary $\beta$-glu inhibitors (*i.e.* L-mal) might provide an effective prophylactic treatment for jaundice related to breast milk ingestion without compromising breast milk intake. Whether or not such *in vitro* observations as we have presented here will have significant effects *in vivo* can only be assessed through appropriately performed clinical trials. We look forward to performing such a trial if adequate support can be secured.

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