Enzymology Research Center, Inc.
332 West Street North, Miltona, MN 56354 U.S.A.
Phone: 1.888.661.1100 - Fax: 1.888.677.1100

AstraZyme® Study

AstraZyme® is a unique combination of proteolytic enzymes with trace minerals (ERC1™) and extracts of Astragalus membranaceus and Panax notoginseng (AstraGin®).

Part 1- The effect of ERC1™ (a proprietary ERC enzyme blend with trace minerals) on the breakdown of protein (bovine hemoglobin) into peptides and amino acids.

Part 2- The effect of AstraGin® on the absorption of peptides and amino acids (derived from ERC1™) in human small intestinal Caco-2 cells.

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1. Abstract

The objectives of the present study are threefold:

1. To evaluate the ability of ERC1™ on bovine hemoglobin digestion.

In the bovine hemoglobin digestion study, ERC1™ hydrolyzed >90% of the hemoglobin to peptides and amino acids in 60 minutes over a wide range of pH. This indicates that ERC1™ works well in human digestive tract. In another time-course digestion test, ERC1™ hydrolyzed greater than 95% of the hemoglobin to peptides and amino acids in acidic environment within 60 minutes and the percent of unhydrolyzed hemoglobin continually decreased as time passed.

2. To observe the effect of AstraGin® on the absorption of the hemoglobin-derived peptides hydrolyzed by ERC1™ in human small intestinal Caco-2 cell monolayers.

In the peptide absorption study, no peptides were absorbed in Caco-2 cells when whole

hemoglobin solution was added to the medium without ERC1™. When the hemoglobin was hydrolyzed by ERC1™, AstraGin® was able to increase the amount of peptides absorption (AUC) by 41% and 83% in 45 minutes at 1X and 10X strength and the rate of absorption by 30% and 66% in 45 minutes at 1X and 10X strength.

3. To observe the effect of AstraGin® on the absorption of the hemoglobin-derived amino acids hydrolyzed by ERC1™ in human small intestinal Caco-2 cell monolayers.
In the amino acids absorption study, very low amount of amino acids were absorbed in Caco-2 cells when whole hemoglobin solution was added to the medium without ERC1™. When the hemoglobin was hydrolyzed by ERC1™, AstraGin® was able to increase the amount of amino acids (AUC) absorption by 70% and 125% with 1X and 10X AstraGin® and the rate of absorption by 61% and 110% in 15 minutes with 1X and 10X strength.

In summary, the study demonstrates that ERC1™ was able to hydrolyze 90% or greater amount of the bovine hemoglobin to peptides and amino acids. AstraGin® was shown to increase the amount as well as rate of absorption of the peptides and amino acids.

AstraZyme® is a proprietary and proven combination of proteolytic enzymes and trace minerals (ERC1™) with extracts of Astragalus membranaceus and Panax notoginseng (AstraGin®).

2. Summary

Table 1. Percent of unhydrolyzed hemoglobin with ERC1™ at indicated pH buffer

Undigested hemoglobin (%)	pH in buffer			
	4	6	8	10
No ERC1™	100.00±0.30			
With ERC1™	3.34±0.22	4.29±0.231	9.76±0.11	9.12±0.40

Table 2. Percent of unhydrolyzed hemoglobin with ERC1™ at indicated time.

With ERC1™ at pH4 buffer		Undigested hemoglobin (%)
Time (min)	0	100.00±0.38
	30	12.17±0.10
	60	4.30±0.02
	90	1.87±0.02
	120	2.04±0.02

Table 3. Transport rate of peptides in 45 minutes

AstraGin® doses	ERC1™	Relative transport rate of peptides in 45 minutes (%)
0X	-	<1
0X	+	100.00 ± 14.24
1X	+	130.38 ± 12.14*,##
10X	+	165.87 ± 12.21*,##

Table 4. Total amount of peptides (AUC) absorbed in Caco-2 cells in 45 minutes

AstraGin® doses	ERC1™	Relative area under curve of peptide absorption (%)
0X	-	<1
0X	+	100.00±15.82
1X	+	141.04±13.48*
10X	+	183.02±13.57*

Table 5. Transport rate of amino acids in 15 minutes

AstraGin® doses	ERC1™	Relative transport rate of amino acids in 15 minutes (%)
0X	-	<1
0X	+	100.00 ± 2.85
1X	+	161.38 ± 13.01**.##
10X	+	209.76 ± 20.33**.##

**p<0.01, when compared to ERC1™ only group
p<0.01, when compared to Blank group (No ERC1™, no AstraGin® added)

Table 6. Total amount of amino acids absorbed in Caco-2 cells in 15 minutes.

AstraGin® doses	ERC1™	Relative area under curve of total amino acids absorption (%)
0X	-	<1
0X	+	100.00±3.81
1X	+	170.36±7.25**.##
10X	+	224.95±8.41**.##

3. Objective

AstraGin® has been validated and demonstrated to enhance the cellular absorption of amino acids, vitamins, and glucose in NuLiv Science's ***In vitro*** and ***In vivo*** studies. Details of the studies are presented in the AstraGin™ product dossier.

The purpose of this study is to assess the effectiveness of ERC1™ on protein digestion (specifically bovine hemoglobin) and AstraGin® on the absorption of hemoglobin-derived peptides and amino acids hydrolyzed by ERC1™ in human small intestine Caco-2 cells.

AstraZyme® is a proprietary and proven combination of proteolytic enzymes and trace minerals (ERC1™) with extracts of Astragalus membranaceus and Panax notoginseng (AstraGin®).

4. Materials & Methods

Protease activity assay

Protease activities of the samples were measured by QuantiCleave™ Protease Assay Kit according to the manufacturer's protocol (Pierce, Rockford, IL). Measure absorbances of wells in a plate reader set to 450nm. For each well calculate the change in absorbance at 450nm (ΔA450) by subtracting the A450 of the blank from that of the corresponding substrate well. This ΔA450 is the absorbance generated by the proteolytic activity of the protease. The protease activity was determined by SPECTRA MAX190 (Molecular Device, USA)

Cell Culture

The Caco-2 cell line was obtained from ATCC (Philadelphia, PA, USA). The Caco-2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco Life Technology), nonessential amino acids, L-glutamine and penicillin/streptomycin. The Caco-2 cells were incubated at 37℃ in a humidified atmosphere containing 5% CO_2. The cells used in the experiments were between passages 10 and 20. Caco-2 cells were subcultured weekly by trypsin and were seeded at a ratio of 1:3 upon reaching 80% confluence. The culture medium was changed every 2–3 days. For the transport experiments, the cells were seeded at a density of $9x10^5$ cells/cm^2 in 6-well filter support inserts with polyethylene membranes (0.4 μm pore size, 24 mm diameter, 4.67 cm^2 growth surface area; Costar, Corning Inc., Corning, NY). The monolayers reached confluence in 3 days after seeding, and the cells were differentiated for at least an additional 14 days prior to the transepithelial transport

experiments. The integrity of the Caco-2 cell monolayers and the tight junctions were monitored before every experiment by determining the transepithelial electrical resistance (TEER) measurements using an epithelial Volt-Ohm Meter (Millicell ERS-2, Millipore, Bedford, MA). Only the Caco-2 monolayers with TEER values higher than 700Ω·cm² were used for the experiments.

Preparation of bovine hemoglobin hydrolysate

Bovine hemoglobin was dissolved at 5% (w/v) in water, and mixed vigorously and filtered through a glass wool filter. Hemoglobin solution was diluted to 4% with 300mM HCl. Immediately before digestion, prepare ERC1™ in sodium acetate buffer, pH4. 2% hemoglobin solution was digested by incubation with 1% (w/w) ERC1™ for 2 h at 37℃ in a orbital incubator during the reaction, aliquots of the hemoglobin and ERC1™ mixture were taken out at various times. Each aliquot was immediately added with ammonium hydroxide to a value of pH 10 and heated in boiling water for 10 min to inactivate the enzyme activity and stop the reaction. The precipitate was removed from the peptic digest by centrifugation (10000 rpm, 4℃, 20 min). The supernatants were lyophilized before further use. Sample mixtures were stored at -30℃.

Tris-Tricine-SDS-PAGE electrophoresis

Tricine-SDS-PAGE was carried to evaluate the peptide profile after ERC1™ treatment. The Tris-Tricine-SDS-PAGE method was carried out according to Schägger & von jagow (1987), using 19.5% separating gel and 10x10 cm glass plates in the Bio-Rad system. Staining was performed according to a standard procedure.

Transepithelial transport studies

After TEER measurement, the differentiated Caco-2 monolayers were gently rinsed twice with Hank's balanced salt solution (HBSS) and equilibrated for 30 min at 37℃. Then the medium was replaced with fresh HBSS containing the peptides derived from bovine hemoglobin hydrolyzed by ERC1™. The transwells were incubated at 37℃ for 120 min and the apical and basolateral medium were sampled at the designated time intervals and analyzed by fluoraldehyde (OPA) peptide assay. During and at the end of the experiments, TEER was measured and data were recorded only from experiments in which TEER was higher than 250Ω·cm²

Fluoraldehyde (OPA) peptide assay

Peptide concentration was measured by the method of fluoraldehyde reaction using bovine serum albumin (BSA) as a standard. BSA (2mg/ml) was digested by incubation with 1% (w/w) trypsin for 72 h at 37℃ in a orbital incubator. The undigested protein was removed by the addition of TCA, followed by centrifugation at 10,000g for 20 min, the peptide content of the supernatant was measured. Peptide concentrations of the samples were measured by the fluoraldehyde (OPA) assay kit according to the manufacturer's protocol (Pierce, Rockford, IL). Briefly, prepare samples, blanks and standards in an opaque plate. Add optimal volume of OPA reagent solution to each well and mix well. Measure the fluorescence at excitation 330-390nm and emission at 436-475nm. The peptide concentrations were determined by Polarstar Galaxy (BMG LABTECH, DE).

MALDI-TOF mass spectrometry

The MALDI-TOF MS experiments were performed on microflex (Bruker Daltonics, USA) equipped with an N_2 laser. Prior to the MS analysis, the dried peptide mixtures were dissolved in ddH_2O, and desalted using C18 Zip-Tip pre-packed micro-columns (Millipore, Bedford, MA, USA) previously equilibrated with aqueous 0.1% TFA (v/v) and eluted with 70% acetonitrile (v/v) containing 0.1% TFA (v/v). The HPLC peaks were directly loaded onto a stainless steel plate together with α-cyano-4-hydroxycynnamic acid matrix and air-dried. The mass spectra were acquired in the positive ion reflector mode by accumulating 400 laser pulses. The external mass calibration was performed with mass peptide standards (Sigma). The spectra were analyzed with the FlexAnalysis version 3.4 (Bruker Daltonics, USA).

Amino acid quantitation analysis

Amino acids concentration was measured by using commercial Menagent test kits (BioVision, USA). Briefly, prepare samples, blanks and standards in an opaque plate. Add optimal volume of amino acid reaction mix solution to each well and mix well. Measure the fluorescence at excitation 535 nm and emission at 590nm. The amino acid concentrations were corrected

5. Results

A. Protease Activity

Figure1. ERC1™ enzymes activity (A) Limit detection of enzymes activity. Enzyme concentrations are plotted using logarithmic scales; (B) Dose effect of enzymes activity.

This ΔA450 is the absorbance generated by the proteolytic activity of ERC1™. When ΔA450 is higher, their proteolytic activity is higher. The Lower limit of activity assay for ERC1™ was 50μg/mL. When ERC1™ concentration <1000 μg/mL, their proteolytic activities proceed at a rate that is dependent of reactant concentration (initial rate of reaction), and when concentration >1000 μg/mL, their proteolytic activities proceed at a rate that is independent of reactant concentration.

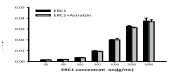

Figure2. Effect of AstraGin® on ERC1™ activity.

Effect of AstraGin® on ERC1™ activity : There was no statistical difference between ERC1™ group and ERC1™ +AstraGin® group. It also means AstraGin® 's involvement is independent of ERC1™ activity.

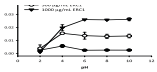

Figure3. Effect of pH on ERC1™ activity. ERC1™ activity at various pH conditions.

100-1000μg/mL of ERC1™ was selected to observe pH effect. Most ERC1™ concentrations have higher enzyme activity in pH4, except for when 1000 μg/mL used. Effect of pH on ERC1™ activity is more noticeable in lower concentrations.

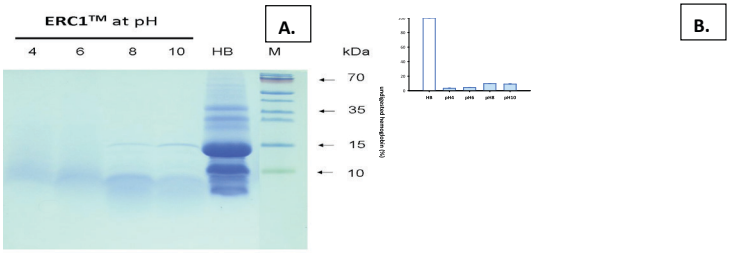

Figure 4. Undigested hemoglobin withERC1™ at indicated pH buffer. (A) Tricine-SDS-PAGE of hemoglobin hydrolysates by ERC1™. 4, pH4; 6, pH6; 8, pH8; 10, pH10; HB, 2% hemoglobin in H_2O; M, pre-stained protein marker; kDa, separated standard protein molecular weight. (B) Quantitative analysis of the results shown in panel.

Table 1. Percent of undigested hemoglobin with ERC1™ at indicated pH buffer.

Undigested hemoglobin (%)	pH in buffer			
	4	6	8	10
No ERC1™	100.00±0.30			
With ERC1™	3.34±0.22**	4.29±0.231**.#	9.76±0.11**.##	9.12±0.40**.##

** p<0.01, when compared to hemoglobin only (No ERC1™) group.
# p<0.05, when compared to hemoglobin+ ERC1™ at pH2 group
p<0.01, when compared to hemoglobin+ ERC1™ at pH2 group

As indicated in Fig.4, after 1 h of incubation at 37℃ of hemoglobin in the presence of 1% (w/w) ERC1™, the hemoglobin bands almost completely disappeared on the electrophoresis gel, especially at acidic buffer, e.g. pH4. The higher subunits of hemoglobin (>25kDa) were completely undetectable. The smaller smeared bands were mainly produced forms of ERC1™'s digestion. ERC1™ adapted well to a wide range of pH, and most high enzymatic activity appeared in acidic environment. Even though there was a 15kDa subunit slightly appeared at pH8 or pH10 buffer when hemoglobin digested by ERC1™, this didn't change the fact that ERC1™ had the highly digestive capacity to digest >90% hemoglobin in 60 minutes over wide range of pH.

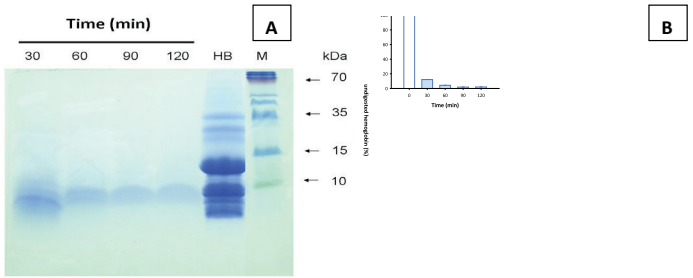

Figure5. Undigested hemoglobin with ERC1™ at indicated time. (A) Tricine-SDS-PAGE of hemoglobin hydrolysates by ERC1™. (B) Quantitative analysis of the results shown in panel.

Table 2. Percent of undigested hemoglobin with ERC1™ at indicated time.

With ERC1™ at pH4 buffer		Undigested hemoglobin (%)
Time (min)	0	100.00±0.38
	30	12.17±0.10**
	60	4.30±0.02**.##
	90	1.87±0.02**.##.††
	120	2.04±0.02**.##.††

** p<0.01, when compared to hemoglobin only group.
p<0.01, when compared to "hemoglobin digestion by ERC1™ for 30min" group.
†† p<0.01, when compared to "hemoglobin digestion by ERC1™ for 60min" group.

As indicated in Fig.5, incubation of hemoglobin in the presence of 1% (w/w) ERC1™ at pH4 buffer, the hemoglobin digestion appeared partial after 30 min with the apparition of bands with smaller molecular weights. The relative percentage of undigested hemoglobin after 30min of ERC1™'s action was approximately 10%. By time pass, the amounts of undigested hemoglobin were decreased. The maximum limitation of ERC1™ was reached after 90 minutes of digestion time. There was no statistical difference between 90 minutes and 120 minutes.

B. Peptide absorption

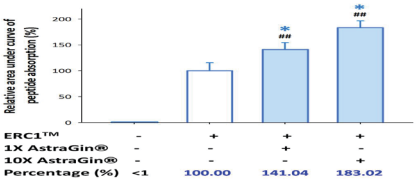

Figure 6. Effect of AstraGin® on the absorption rate of peptides produced from ERC1™ in 45 minutes.

Table 3. Relative transport rate of peptides in 45 minutes.

AstraGin® doses	ERC1™	Relative transport rate of peptides in 45 minutes (%)
0X	-	<1
0X	+	100.00 ± 14.24
1X	+	130.38 ± 12.14*.##
10X	+	165.87 ± 12.21*.##

*p<0.05, when compared to ERC1™ only group.
p<0.01, when compared to Blank group (No ERC1™, no AstraGin® added)

The differentiated Caco-2 cell monolayers were pretreated with AstraGin® for 24h, and then incubated for 120 min with the peptides derived from bovine hemoglobin hydrolyzed by ERC1™. During the incubation, the medium from the basolateral compartments were collected at designated time intervals and analyzed by fluoraldehyde (OPA) peptide assay. Our results indicated that AstraGin® increased the initial absorption rate of peptides derived from bovine hemoglobin hydrolyzed by ERC1™ by 30.39% and 65.87% with 1X and 10X AstraGin® respectively when compared to the ERC1™ only group. Notably, it was not feasible to observe the peptide transport phenomenon when hemoglobin without ERC1™'s action. This was due to intact protein too large to across the intestinal membrane. Without enzyme digestion, it was difficult to transport large size of protein into the intestinal cells.

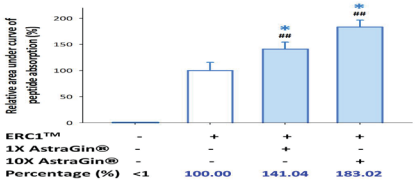

Figure 7. Effect of AstraGin® on the total quantity of peptides derived from bovine hemoglobin hydrolyzed by ERC1™ in 45 minutes

Table 4. Total amount of peptides absorbed by Caco-2 cells in 45 minutes.

AstraGin® doses	ERC1™	Relative area under curve of peptide absorption (%)
0X	-	<1
0X	+	100.00±15.82
1X	+	141.04±13.48*
10X	+	183.02±13.57*

*p<0.05, when compared to ERC1™ only group
p<0.01, when compared to Blank group (No ERC1™, no AstraGin® added)

When bovine hemoglobin was not hydrolyzed by ERC1™, the total quantity of peptides absorbed in Caco-2 cells was minimum with or without AstraGin®. When bovine hemoglobin was hydrolyzed by ERC1™, the total quantity of peptides absorbed in Caco-2 cells was 41% and 83% with 1X and 10X AstraGin® respectively.

C. Amino Acid Absorption

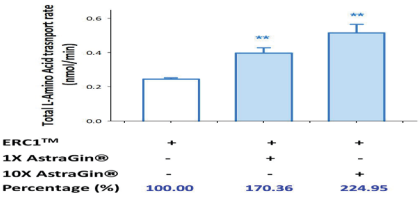

Figure 8. Effect of AstraGin® on the absorption rate of bovine hemoglobin-derived amino acids hydrolyzed by ERC1™ in 15 minutes.

Table 5. Relative transport rate of amino acids in 15 minutes.

AstraGin® doses	ERC1™	Relative transport rate of amino acids in 15 minutes (%)
0X	-	<1
0X	+	100.00 ± 2.85
1X	+	161.38 ± 13.01**,##
10X	+	209.76 ± 20.33**,##

**p<0.01, when compared to ERC1™ only group
p<0.01, when compared to Blank group (No ERC1™, no AstraGin® added)

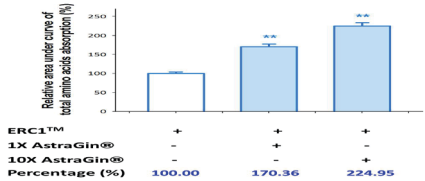

Figure 9. Effect of AstraGin® on the absorption of total amount of bovine hemoglobin-derived amino acids hydrolyzed by ERC1™ in 15 minutes

Table 6. Total amount of amino acids absorbed in Caco-2 cells in 15 minutes.

AstraGin® doses	ERC1™	Relative area under curve of total amino acids absorption (%)
0X	-	<1
0X	+	100.00±3.81
1X	+	170.36±7.25**.##
10X	+	224.95±8.41**.##

The differentiated Caco-2 cell monolayers were pretreated with AstraGin® for 24 hours, and then incubated for 120 min with the bovine hemoglobin-derived amino acids hydrolyzed by ERC1™. During the incubation, the medium from the basolateral compartments were collected at designated time intervals and analyzed by L-amino acid quantitation fluorometric kit. In the amino acids absorption study, very low amount of amino acids were absorbed in Caco-2 cells when whole hemoglobin solution was added to the medium without ERC1™. After bovine hemoglobin was hydrolyzed by ERC1™, AstraGin® was able to increase the total amount of amino acids (AUC) absorption by 70% and 125% with 1X and 10X AstraGin® in 45 minutes and the rate of absorption by 61% and 110% with 1X and 10X AstraGin® in 15 minutes.

6. Discussion

In the protease activity assay, the detection limit for ERC1™ was 50µg/ml. When enzyme concentration was <1000 µg/mL, ERC1™ proceeded at a rate that was dependent of reactant concentration (initial rate of reaction). Optimal concentrations of ERC1™ were selected between 100-1000µg/mL for further experiments. When AstraGin® was added to ERC1™ in bovine hemoglobin hydrolysis, AstraGin®'s involvement is independent of ERC1™ activity. The enzyme activity between ERC1™ and ERC1™ +AstraGin® had no statistical differences. However, AstraGin® is safe to be included in ERC1™. The pH study indicated ERC1™ worked well in a wide range of pH and most high enzymatic activity appeared in acidic environment. This characteristic allows ERC1™ to work well in human digestive system.

By gel electrphoresis, we observed that hemoglobin bands almost completely disappeared on the electrophoresis gel when ERC1™ was involved in hemoglobin digestion. The smaller smeared bands were mainly produced forms of ERC1™'s digestion. ERC1™ adapted well to a wide range of pH, and ERC1™ had high digestive capacity to digest >90% of the hemoglobin in 60 minutes over wide range of pH. In another time-course digestion test, we also observed that ERC1™ digested greater than 95% of the hemoglobin within 60 minutes, and percent of the undigested hemoglobin continually decreased as time pass.

Bovine hemoglobin was not absorbed in Caco-2 cells due to its large size. ERC1™ hydrolyzed bovine hemoglobin to peptides and amino acids by cleaving the peptide bonds. After hydrolysis by ERC1™, AstraGin® was shown to increase the total amount of absorption (AUC) of bovine hemoglobin-derived peptides by 41% and 83% in 45 minutes with 1X and 10X strength and the rate of absorption by 30% and 66% in 45 minutes at 1x and 10x strength.

AstraGin® was also shown to increase the total amount of absorption (AUC) of bovine hemoglobin-derived amino acids by 70% and 125% in 15 minutes with 1X and 10X strength and the rate of absorption by 61% and 110% in 15 minutes at 1x and 10x strength.

In conclusion, this study demonstrated that AstraGin® significantly increased the amount and rate of transport of amino acids and peptides across the Caco-2 cell monolayer. In our previous studies, we demonstrated that AstraGin® conferred significant and parallel alterations in mRNA and carrier transport protein levels to increase various nutrients absorption in Caco-2 cells. This study demonstrated that adding AstraZyme® which is a combination of ERC1™ and AstraGin® can not only effectively breakdown proteins but also increase the bioavailability and absorption of peptides and amino acids in human intestinal cells.

7. References

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