Enzymology Research Center, Inc.
332 West Street North Miltona, MN 56354
888-661-1100 Office 888-677-1100 Fax

Effect of AstraGin® and AstraZyme® on the absorption of amino acids and BCAA derived from ECO hemp seed protein (Hempco Canada) in human Caco-2 cells

Table of Contents

1. Abstract

The purpose of this study is to assess the effect of AstraGin® on the absorption of ECO hemp seed protein (from Hemp Canada) derived amino acids and BCAA in human small intestine Caco-2 cells. Three digestive enzymes, T (trypsin+pepsin+pancreatin), A (AstraZyme®), and T+A(AstraZyme®), were used to digest the ECO hemp seed protein for 2h and AstraGin® was added in differentiated Caco-2 cells for 24h prior to the studies.

AstraGin® increased total quantity of amino acids absorption in Caco-2 cells by 23%, 23% and 43% for ECO hemp seed protein treated with T, A (AstraZyme®), and T+A (AstraZyme®) digestive enzymes. AstraGin® increased total quantity of BCAA absorption in Caco-2 cells by 30%, 32% and 69% for ECO hemp seed protein treated with T, A (AstraZyme®), and T+A (AstraZyme®)digestive enzymes.

In summary, results of the study indicate AstraZyme® broke down (digested) more hemp protein into amino acids. AstraGin® increased the amino acids and BCAA absorption from hemp seed protein that were digested by T+A (AstraZyme®) digestive enzymes.

2. Summary

Table 1. Percent of amino acids and BCAA in ECO hemp seed protein digested with T, A(AstraZyme®), and T+A(AstraZyme®) digestive enzymes with T digestive enzyme treated hemp seed protein as the control

Amino acids	Group		
	T(Trypsin, pepsin and pancreatin)	A(AstraZyme ®)	T+A(AstraZyme®)
Total amino acids	100.0±10.3	23.1±1.0**	157.2±18.1**
BCAA	100.0±3.4	42.9±1.9**	142.9±8.1*

*p<0.05, when compared to control (T) group

** p<0.01, when compared to control (T) group

Table2. Percent of amino acids absorbed in Caco-2 cells in 60 minutes.

Treatment	Total quantity of amino acids (%)		
	T(Trypsin, pepsin and pancreatin)	A(AstraZyme®)	T+A(AstraZyme®)
Control	100.0±16.8	100.0±13.1	100.0±22.6
AstraGin®	122.7±16.4*	123.0±20.0**	143.1±22.1*

*p<0.05, when compared to corresponded control group

** p<0.01, when compared to corresponded control group

Table3. Percent of BCAA absorbed in Caco-2 cells in 60 minutes.

Treatment	Total quantity of amino acids (%)		
	T(Trypsin, pepsin and pancreatin)	A(AstraZyme®)	T+A(AstraZyme®)
Control	100.0±20.5	100.0±16.3	100.0±21.6
AstraGin®	130.4±18.3*	132.1±18.1**	169.2±20.5*

*p<0.05, when compared to corresponded control group

** p<0.01, when compared to corresponded control group

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.
AstraZyme® has been validated and demonstrated to enhance the breakdown and absorption rates and quantities of peptides and amino acids in ERC's studies.
The purpose of this study is to determine the percent of ECO hemp seed protein that is broken down to amino acids and BCAA by the T, A(AstraZyme®), T+A(AstraZyme®) digestive enzymes and the effect of AstraGin® on the absorption of these amino acids and BCAA in human small intestine Caco-2 cells.

4. Materials & Methods

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 1:3upon 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 • cm^2 were used for the experiments.

Preparation of hemp seed protein isolate (HPI)

Hemp seed protein isolate (HPI) was prepared according to a literature method. ECO raw organic hemp seed protein powder was suspended in deionized water (1:20, w/v) at RT under stirring and the mixture was adjusted to pH 10.0 with 2 N NaOH solubilize the proteins while stirring at 37 °C. After 120 min, samples were centrifuged at 7000g for 60 min at 4 °C. The pellet was discarded, the supernatant was filtered with cheese-cloth and adjusted to pH 5.0 with 2 N HC to precipitate the proteins, and the precipitate was collected by centrifugation (7000g, 60 min). The precipitate was then resuspended in deionized water, adjusted to pH 7.0 with 2 M NaOH and freeze-dried to obtain the HPI. Protein concentrations of the HPI were determined using BCA assay kit.

Preparation of HPI hydrolysate

The HPI samples were each dispersed (10 mg/mL) in Tris/HCl buffer, pH 8.0. Three typical digestive enzymes: pepsin, trypsin, and pancreatin were used to mimic the gastrointestinal digestion. HPI was digested by three digestive enzymes (T), or AstraZyme® (A) or a combination of three digestive enzymes with AstraZyme® (T+A). The enzyme solution (50 mg/mL in 30 mM NaCl) was added in a 1:50 enzyme/hemp seedprotein ratio (w/w). The mixture was incubated for 2 h, and the enzyme was inactivated. Each digestion was stopped by holding at 95 °C for 10 min to ensure a complete inactivation of residual enzyme activity. All digestion processes were

performed at 37 °C, and all obtained hydrolysates were freeze-dried, then stored at -30°C until absorption experiment.

SDS-PAGE electrophoresis

SDS-PAGE was carried to evaluate the protein profile after HPI extraction or HPI digestion. The efficiency of protein extraction was checked by SDS-PAGE electrophoresis using 10% separating gel and 4% stacking gel. The samples were then heated for 5 min in boiling water before electrophoresis. Each sample (10 μL) was applied to each lane. The gel was stained with 0.25% Coomassie brilliant blue(R-250) in methanol–water (1:1), and destained in 7% acetic acid in methanol–water (1:1). Staining was performed according to a standard procedure.

Transepithelial transport studies

After TEER measurement, the differentiated Caco-2 monolayers were gently rinsed twice with phosphate-buffered saline (PBS, pH 7.4) supplemented with 25 mM glucose, 10 μM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-GCM). Prior to absorption studies, HPI hydrolysates were resuspended in PBS-GCM, then desalted by Sep-Pak C18 cartilages (Waters). The transport experiment was initiated by replacing the incubation solution on the apical side with each HPI hydrolysates. The transwells were incubated at 37℃ for 120 min and the basolateral mediums were sampled at the designated time intervals. The end of the experiments, TEER was measured and data were recorded only from experiments in which TEER was higher than 250Ω • cm^2.Results are expressed as the total amino acids transport across (nanomoles per minute) across the Caco-2 monolayers in mean ± SD (n = 3-5). Differences between means of groups were assessed by the paired t-test.

Total amino acids assay

The amino acids contents were assayed by Biovision K639-100L-Amino Acid Quantitation Colorimetric/Fluorometric Kit according to the manufacturer's protocol.

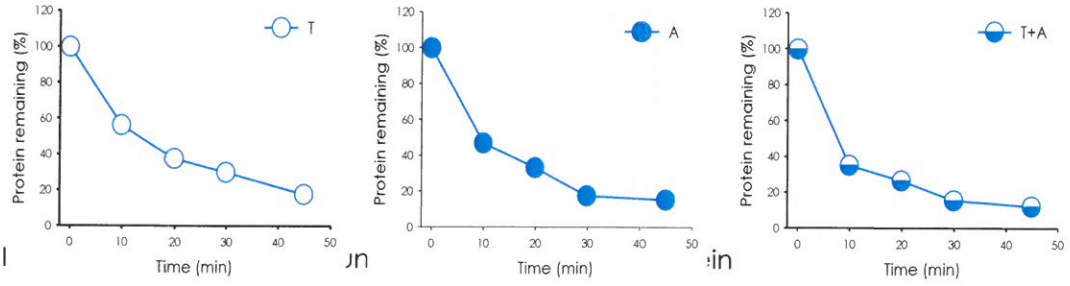
Branched chain amino acid assay

BCAA contents were assayed by Biovision K564-100 branched chain amino acid Colorimetric assay Kit according to the manufacturer's protocol.

5. Results

5.1. Percent of ECO hemp seed protein isolate undigested

5.1.1. Percent of ECO hemp seed protein isolate undigested



digested by T, A (AstraZyme®), T+A(AstraZyme®) digestive enzymes for 2h.Samples were collected at designated intervals for gel electrophoresis.

Table1.Percent of ECO hemp seed protein isolate (HPI)left after 2h incubation with T, A(AstraZyme®), and T+A(AstraZyme®) digestive enzymes.

Time (min)	Protein remaining (%)		
	T	A(AstraZyme®)	T+A(AstraZyme®)
0	100.0±2.7	100.0±1.6	100.0±1.4
10	56.3±4.4	47.0±0.5**	35.2±1.9**
20	37.7±1.4	33.3±2.9	26.6±0.9**
30	30.0±0.6	17.6±0.7**	15.6±0.5**
45	17.8±0.9	15.4±1.1	12.4±1.1**

*p<0.05, when compared to control (T) group

** p<0.01, when compared to control (T) group

According to the results shown in SDS-PAGE, there are many types of protein in HPI. We observed major proteins appeared differentially between three groups (MW 35-45 kDa around). Three common digestive enzymes: trypsin, pepsin and pancreatin were used to mimic the gastrointestinal digestion and were used as the control (T). Table 1 showed ECO hemp seed protein was easily digested because more than 50% of it was digested in most groups within 10min, and about 15% was not digested after 45min. The T+A(AstraZyme®) digestive enzymes have the highest digestive capacity in each time point than either the T or A(AstraZyme®) digestive enzyme, especially >85% digestion of hemp proteins in 30 minutes.

5.1.2. Total amino acids and BCAA contents after enzymes digestion

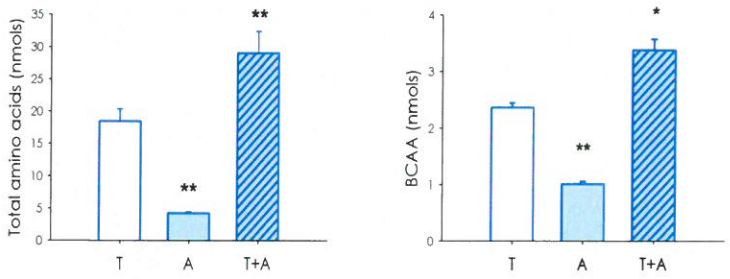


Figure 2. Equal amounts of hemp seed protein isolates (HPI) were digested by T, A(AstraZyme®), T+A(AstraZyme®) digestive enzymes. After 2h incubation, total amino acids and BCAA contents were quantified.

Table 2. Percent of ECO hemp seed protein broken down to amino acids and BCAA by digestive enzymes T, A(AstraZyme®), and T+A(AstraZyme®) with T as the control.

Amino acids	Group		
	T(Trypsin, pepsin and pancreatin)	A(AstraZyme®)	T+A(AstraZyme®)
Total amino acids	100.0±10.3	23.1±1.0**	157.2±18.1**
BCAA	100.0±3.4	42.9±1.9**	142.9±8.1*

*p<0.05, when compared to control (T) group

** p<0.01, when compared to control (T) group

When hemp protein is digested by three digestive enzymes with AstraZyme®, total amino acids and BCAA contents are markedly elevated than in control group. It seems AstraZyme® supplement can be complementary for these physiological enzymes to achieve comprehensive protein digestion.

5.2. AstraGin® on the absorption of ECO hemp seed protein isolate derived amino acids in Caco-2 cells after 24 hour pre-treatment with AstraGin®

5.2.1. AstraGin® on amino acids absorption

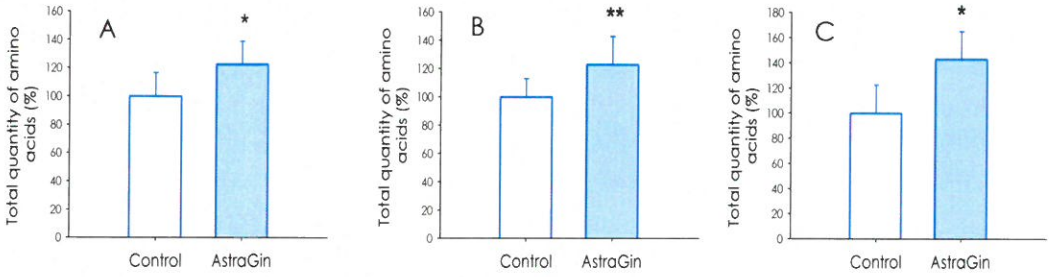


Figure 3. AstraGin® on amino acids absorption in Caco-2 cells after 24 hour pre-treatment with AstraGin®.

Table3. Amino acids absorption in Caco-2 cells in 60 minutes.

Treatment	Total quantity of amino acids (%)		
	T(Trypsin, pepsin and pancreatin)	A(AstraZyme®)	T+A(AstraZyme®)
Control	100.0±16.8	100.0±13.1	100.0±22.6
AstraGin®	122.7±16.4*	123.0±20.0**	143.1±22.1*

*p<0.05, when compared to corresponded control group

** p<0.01, when compared to corresponded control group

5.2.2AstraGin® on BCAA absorption in Caco-2 cells after 24 hour pre-treatment with AstraGin®

5.2.2. AstraGin® on BCAA absorption

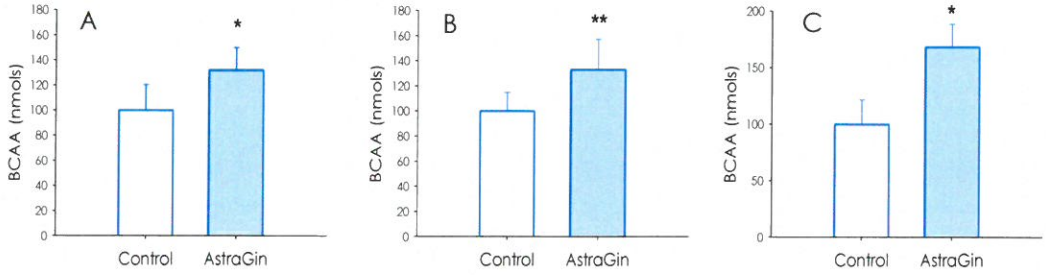


Figure 4. AstraGin® on the absorption of BCAA in Caco-2 cells after 24 hour pre-treatment with AstraGin®.

Table4. Total quantity BCAA absorbed in Caco-2 cells in 60 minutes.

Treatment	Total quantity of amino acids (%)		
	T(Trypsin, pepsin and pancreatin)	A(AstraZyme®)	T+A(AstraZyme®)
Control	100.0±20.5	100.0±16.3	100.0±21.6
AstraGin®	130.4±18.3*	132.1±18.1**	169.2±20.5*

*p<0.05, when compared to corresponded control group

** p<0.01, when compared to corresponded control group

6. Discussion

Hemp seed contains 35.5% oil, 24.8% protein, 20–30% carbohydrates, 27.6% total fiber (5.4% digestible and 22.2% non-digestible fibers) and 5.6% ash. Additionally, the concentration of the main anti-nutritional factors, such as phytic acid, condensed tannins, and trypsin inhibitors are low. There is an increasing attention and interest for hempseed protein owing to its digestibility and essential amino acid composition. Aiello et al., 2016 reported the proteome of hemp seeds, cataloguing 181 expressed proteins in defatted flour.

Three common digestive enzymes: trypsin, pepsin and pancreatin were used to mimic the gastrointestinal digestion, set as control group. In Table 1, we observed hemp proteins were easily digested by all three digestive enzymes. When comparing the specific proteins digestion efficacy between trypsin+pepsin+panceatin (the control) and AstraZyme®, we found AstraZyme® had a higher protein digestibility in each time point. This indicates that AstraZyme® can effectively break down hempseed protein in the digestive tract. It is worth noting that combining AstraZyme® with trypsin+pepsin+pancreatin displayed the highest digestive capacity. We know

food allergy prevalence numbers are still on the rise. Digestion and digestibility of proteins thus critically affect the risk of food allergy. In ideal condition, our bodies can produce sufficient digestive enzymes to digest protein. But dietary habits, food availability and life-style factors all affect the digestive enzymes. Optimal digestive enzymes supplement can properly break down protein and extract the nutrients.

The small intestine is the most important site of absorption of nutrients in mono gastric mammals. The small intestine and particularly the duodenum serve this purpose because the inner surface area is greatly increased by folding of the epithelium and the presence of villi, tiny fingerlike projections extending into the intestinal lumen. The large surface area is increased at the brush borders. Each villus contains anarteriole and a venole together with a drainage tube of the lymphatic system, a lacteal. Thus, nutrients pass across the epithelial cells and enter either blood capillaries or the lymphatic system. Through the blood, amino acids are delivered to all tissues, where they serve as building blocks for protein synthesis, as precursors for a wide variety of bioactive molecules, and as energy metabolites.

Brush border and basolateral membranes are crossed by amino acids and di-tripeptides by passive (facilitated or simple diffusion) or active (Na+ or H+ co-transporters) pathways. The small intestine has a high capability to absorb free amino acids and small peptides. Most L-amino acids can be transported across the epithelium against a concentration gradient. Although the requirement for concentrative transport *in vivo* is not obvious, since luminal concentrations are usually higher than the plasma level 0.1-0.2 mM. It was recognized that amino acid transport systems accept groups of amino acids rather than individual amino acids. Such as the neutral amino acid transporters (system L) prefers leucine and other large hydrophobic neutral amino acids, and system A prefer alanine and other small and polar neutral amino acids.

From the results shown in Fig.3 & Fig.4, we know AstraGin® plays a comprehensive role in enhancing amino acids and especially BCAA absorption. The results are also consistent with our previous studies (Please refer to AstraGin® product dossier). We have demonstrated that AstraGin® increases arginine, tryptophan and leucine absorption. In this study, AstraGin® also displays its ability to enhance total amino acids absorption. Notably, AstraGin® increases BCAA absorption markedly greater than total amino acids. We know AstraGin® increases the absorption of many nutrients through their specific transporters, such as amino acids transporters. The possible reason for this phenomenon can be explained by the competition of amino acids for specific transporter(s). When many amino acids are waiting to be

transported, the priority depends on the amino acid polarity and affinity with the transport system. There exists a competition for the same group of amino acids during intestinal absorption. In total amino acids absorption, there are many transport systems responsible for the absorption of a group of amino acids. We know AstraGin® increases amino acid absorption when they are digested by trypsin+pepsin+pancreatin and AstraZyme®. It is assumed the synergistic effect is due to each digestive enzyme's superior protein digestion, and its ability to bring about different amino acid profiles. This also means the combination of different digestive enzymes may result in digesting more types of protein in hempseed protein. The more completely digested the hempseed protein, the greater AstraGin®'s ability to increase the absorption.

L-type amino acid transporters (System L) prefer branched-chain and aromatic amino acids, including neurotransmitter precursors. The kinetics of isoleucine, leucine, and valine transport in *Escherichia coli* K-12 has been analyzed as a function of substrate concentration. Isoleucine, leucine, and valine are substrates of this transport system and their apparent K_m values are either 10^{-8}, 2×10^{-8}, or 10^{-7} M, higher Km means low affinity for transport system. With higher affinity for leucine and isoleucine have competition advantage for the transport system. From our BCAA absorption study, we know AstraGin® increased BCAA absorption derived from T, A(AstraZyme®), and T+A(AstraZyme®) digestive enzymes, especially the T+A(AstraZyme®) digestive enzymes. Among three branched amino acids, leucine has a higher content in hemp amino acids composition and a higher affinity for its transporter. It is reasonable to assume leucine is the major BCAA absorbed in the studies. Although we have not studied the effect of AstraGin® on System L, we think it is possible to affect this protein expression or activity in term of our previous studies. Amino acids profiles largely differ among plant-based proteins with leucine contents ranging from 5.1% for hemp to 13.5% for corn protein, compared to 9.0% for milk, 7.0% for egg, and 7.6% for muscle protein. This emphasizes again AstraGin® can be complementary for BCAA absorption in hemp protein.

Taken together, based on innate talent of hemp seed protein, containing all nine essential amino acids, implanting efficiently protein digestion with AstraZyme® and AstraGin®'s absorptive ability will bring us many health benefits.

7. References

1. Proteomic characterization of hempseed (Cannabis sativa L.).J Proteomics. 2016;147:187-1962.
2. Structural and functional properties of hemp seed protein products.J Food Sci. 2014;79(8):C1512-21.
3. New ACE-Inhibitory peptides from hemp seed (Cannabis sativa L.) Proteins. J Agric Food Chem. 2017;65(48):10482-10488.
4. Exploration of potentially bioactive peptides generated from the enzymatic hydrolysis of hempseed proteins. J Agric Food Chem. 2017;65(47):10174-10184.
5. Structural and antihypertensive properties of enzymatic hemp seed protein hydrolysates. Nutrients. 2015;7(9):7616-32.
6. Absorption and Transportation of Amino acids in animals: A Review. JEAS 2016; 9:96-109.

8. Supplement

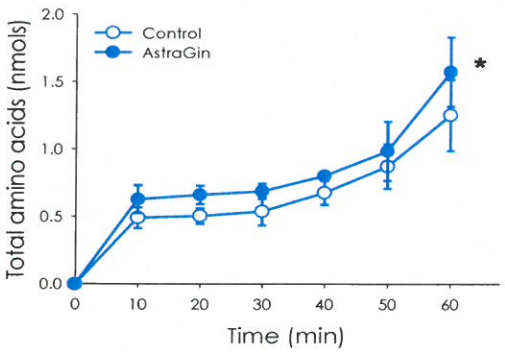


Figure5. Effect of AstraGin® on total amino acids absorption in Caco-2 cells between 0-60 min after 24h treatment. ECO protein isolate are digested by three digestive enzymes, trypsin, pepsin and pancreatin for 2h, and then added into Caco-2 for amino acids study.

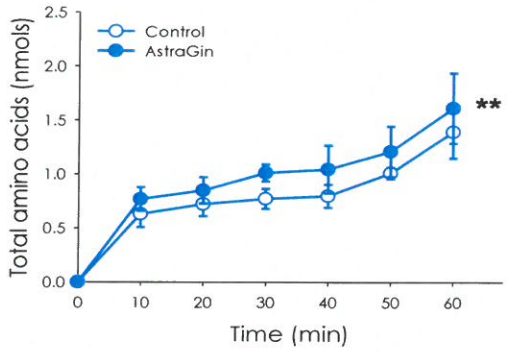


Figure6. Effect of AstraGin® on total amino acids absorption in Caco-2 cells between 0-60 min after 24h treatment. ECO protein isolate are digested by AstraZyme® for 2h, and then added into Caco-2 for amino acids study.

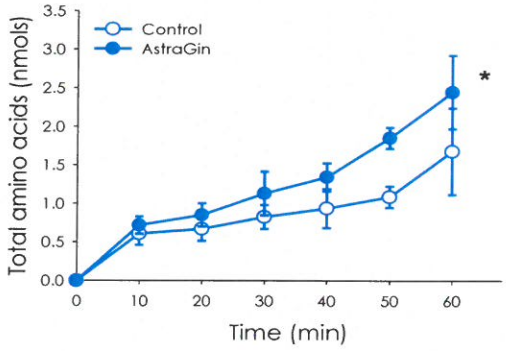


Figure7. Effect of AstraGin® on total amino acids absorption in Caco-2 cells between 0-60 min after 24h treatment. ECO protein isolate are digested by trypsin, pepsin, pancreatin and AstraZyme® for 2h, and then added into Caco-2 for amino acids study.

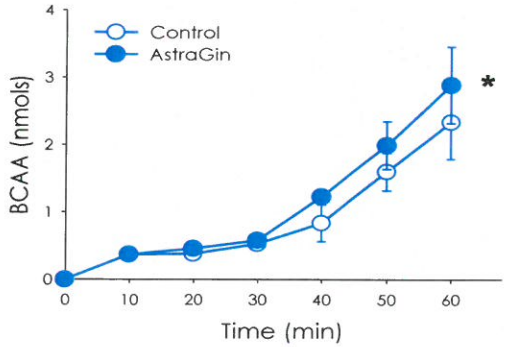


Figure8. Effect of AstraGin® on BCAA absorption in Caco-2 cells between 0-60 min after 24h treatment. ECO protein isolate are digested by three digestive enzymes, trypsin pepsin and pancreatin for 2h, and then added into Caco-2 for BCAA study.

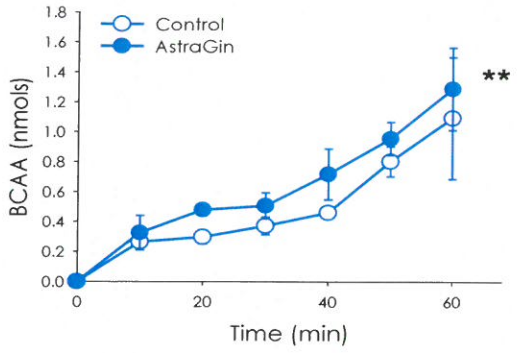


Figure9. Effect of AstraGin® on BCAA absorption in Caco-2 cells between 0-60 min after 24h treatment. ECO protein isolate are digested by AstraZyme® for 2h, and then added into Caco-2 for BCAA study.

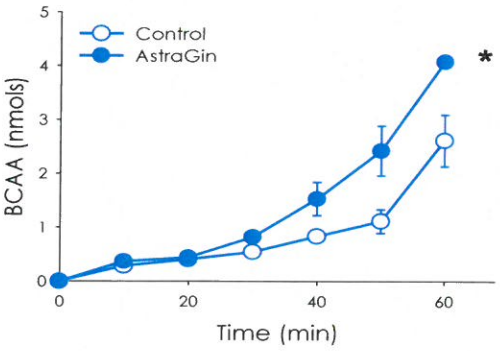


Figure10. Effect of AstraGin® on total amino acids absorption in Caco-2 cells between 0-60 min after 24h treatment. ECO protein isolate are digested by trypsin, pepsin, pancreatin and AstraZyme® for 2h, and then added into Caco-2 for amino acids study.

AstraGin® is a registered trademark of NuLiv Science USA, Inc.

AstraZyme® is a registered trademark of Enzymology Research Center, Inc.

Enzymology Research Center, Inc.
332 West Street North Miltona, MN 56354
888-661-1100 Office 888-677-1100 Fax