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

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

In summary, results of the studies indicate AstraZyme™ was able to digest more proteins in ECO hemp seed protein isolate. AstraGin® was also shown to significantly increase the amino acids and BCAA absorption in hemp seed protein isolate digested with T+A digestive enzymes.

2. Summary

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

Amino acids	Group		
	Trypsin, pepsin and pancreatin (T)	AstraZyme™ (A)	T+A
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

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

Treatment	Total quantity of amino acids (%)		
	Trypsin, pepsin and pancreatin	AstraZyme™	Trypsin, pepsin, pancreatin and 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

Table 3. Percent of BCAA absorbed in Caco-2 cells in 60 minutes.

Treatment	Total quantity of amino acids (%)		
	Trypsin, pepsin and pancreatin	AstraZyme™	Trypsin, pepsin, pancreatin and 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.

The purpose of this study is to determine the percent of ECO hemp seed protein isolate that are broken down to amino acids and BCAA by the T, A, and T+A 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°C 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 HClto 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

seed protein 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 $CaC1_2$ and 1 mM $MgC1_2$ (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°C 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 $250\Omega \cdot cm^2$. 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 undigested ECO hemp seed protein isolate

5.1.1. Percent of ECO hemp seed protein isolate undigested

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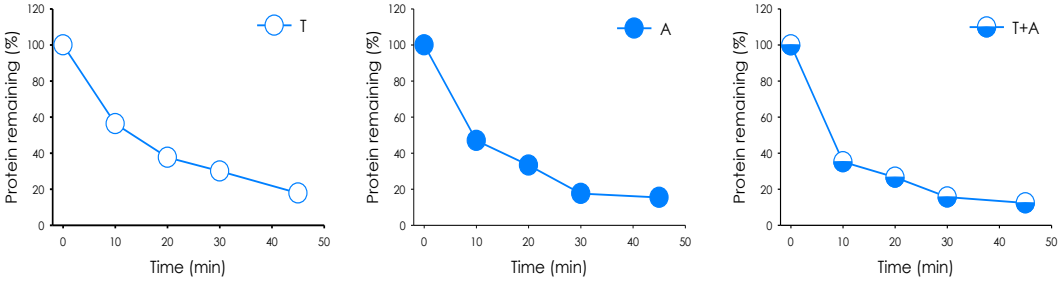

Figure 1. Three equal amount of ECO hemp seed protein isolates (HPI) were each digested by T, A, T+A digestive enzymes for 2h. Samples were collected at designated intervals for gel electrophoresis.

Table 1. Percent of ECO hemp seed protein isolate (HPI) undigested after 2h incubation with T, A, and T+A digestive enzymes.

Time (min)	Protein remaining (%)		
	T	A	T+A
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. Different proteins were found from the hemp seed protein isolate treated with T, A, T+A digestive enzymes (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 isolate was easily digested because more than 50% of it was digested in most groups within 10min, and about 15% remained undigested after 45min. The T+A digestive enzymes had the highest digestive capacity in each time point than either the T or A digestive enzyme, especially >85% digestion of hemp proteins in 30 minutes.

5.1.2. Total amino acids and BCAA contents after enzymes digestion

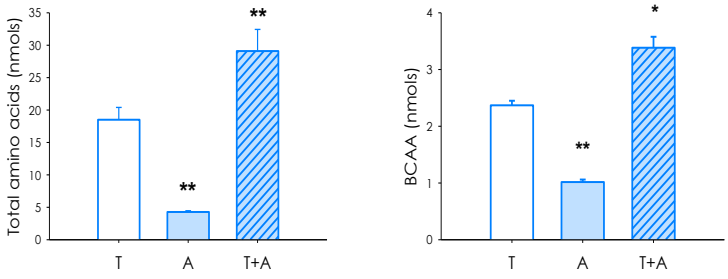

Figure 2. Equal amounts of hemp seed protein isolates (HPI) were digested with T, A, T+A digestive enzymes. After 2h incubation, total amino acids and BCAA contents were quantified.

Table 2. Percent of ECO hemp seed protein broken down to amino acids and

BCAA with T, A, and T+A digestive enzymes with T as the control

Amino acids	Group		
	Trypsin, pepsin and pancreatin (T)	AstraZyme™ (A)	T+A
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 seed protein isolate was digested with T+A digestive enzymes, total quantity amino acids and BCAA were markedly elevated comparing to either the T or A digestive enzymes. This suggests that the combination of T and AstraZyme™ digestive enzymes was able to significantly increase the degree of digestion of hemp seed protein isolate.

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

5.2.1. AstraGin® on amino acids absorption

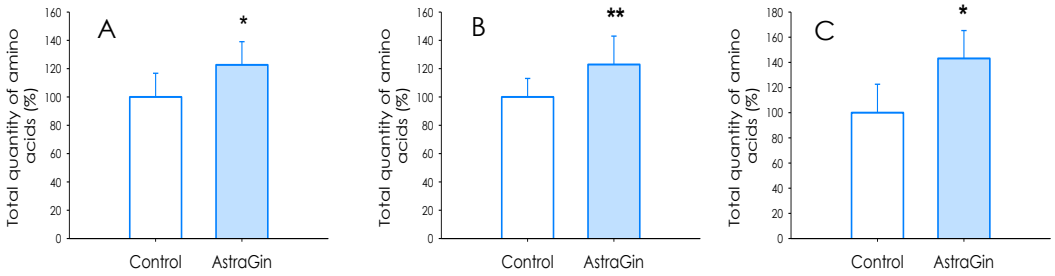

Figure 3. AstraGin® on amino acids absorption in Caco-2 cells after 24h pre-treatment with AstraGin®.

Table 3. Amino acids absorption in Caco-2 cells in 60 minutes.

Treatment	Total quantity of amino acids (%)		
	Trypsin, pepsin and pancreatin	AstraZyme™	Trypsin, pepsin, pancreatin and 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

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

5.2.2. AstraGin® on BCAA absorption

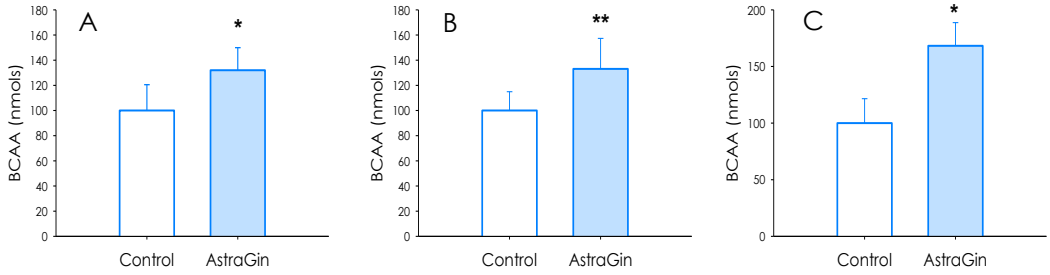

Figure 4. AstraGin® on the absorption of BCAA in Caco-2 cells after 24h pre-treatment with AstraGin®.

Table 4. Total quantity BCAA absorbed in Caco-2 cells in 60 minutes.

Treatment	Total quantity of amino acids (%)		
	Trypsin, pepsin and pancreatin	AstraZyme™	Trypsin, pepsin, pancreatin and 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

Hempseed 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 anti-nutritional factors, such as phytic acid, condensed tannins, and trypsin inhibitors are low. There is an increasing attention and interest in hemp seed 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 hemp seed protein in the digestive tract. Ii is worth noting that combining AstraZyme™ with

trypsin+pepsin+pancreatin displayed the highest digestive capacity. We know food allergy prevalence numbers are 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 supplementation can properly break down protein, increase nutrients bioavailability and minimize allergy.

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 acid. 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 displayed its ability to enhance total amino acids absorption. Notably, AstraGin® increases BCAA absorption markedly greater than total amino acids. We know AstraGin® increased 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 more amino acids absorption when they are digested by trypsin+pepsin+pancreatin and AstraZyme™. It is assumed the synergistic effect is due to each digestive enzyme has it superiority on specific protein digestion, and then bring about different amino acids profile. This also means combination of different digestive enzymes may result in digesting more types of protein in hemp seed protein. More complete digestion on hempseed protein, greater is the AstraGin®'s ability to increase the absorption of their amino acids.

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. Lower K_m value means higher affinity for transport system. Having higher affinity, leucine and isoleucine have competitive advantage for the transport system and higher absorption. From our BCAA absorption study, we observed that AstraGin® increased the absorption of BCAA derived from T, A, and T+A digested hemp seed protein isolate, especially from the hemp seed protein isolate digested with T+A digestive enzymes. Among three branched amino acids, hemp seed protein has higher leucine content and higher affinity for its transporter. It is reasonable to assume that 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 the protein expression or activity in view of our previous studies. Amino acids profiles differ greatly in leucine content among plant-based proteins, ranging from 5.1% for hemp to 13.5% for corn protein, 9.0% for milk, 7.0% for egg, and 7.6% for muscle protein. The studies showed that AstraGin® significantly increased the absorption of BCAA in hemp seed protein.

Taken together, with the protein profile of hemp seed protein with all nine essential amino acids and high leucine content, the combination of AstraZyme™ and AstraGin®'s will yield the highest bioavailability of the essential amino acids and leucine in hemp seed protein.

7. References

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6. Absorption and Transportation of Amino acids in animals: A Review. JEAS 2016; 9:96-109.

8. Supplement

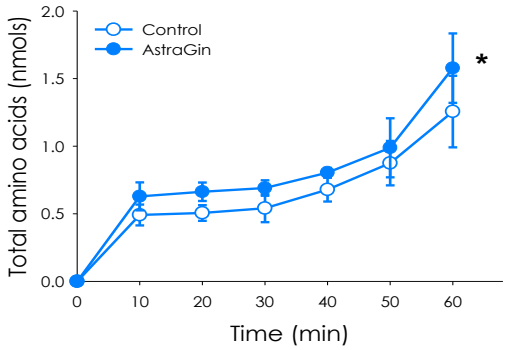

Figure 5. Effect of AstraGin® on total amino acids absorption in Caco-2 cells between 0-60 min after 24h treatment. ECO protein isolate are digested by three digestive enzymes, trypsin, pepsin and pancreatin for 2h, and then added into Caco-2 for amino acids study.

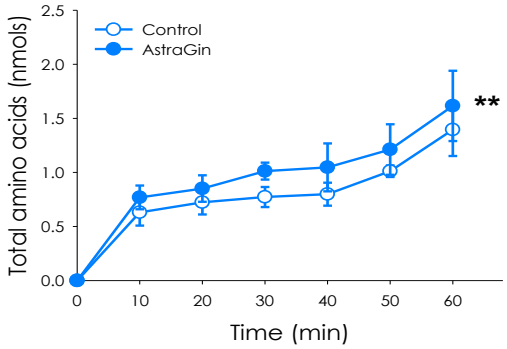

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Figure 6. Effect of AstraGin® on total amino acids absorption in Caco-2 cells between 0-60 min after 24h treatment. ECO protein isolate are digested by AstraZyme™ for 2h, and then added into Caco-2 for amino acids study.

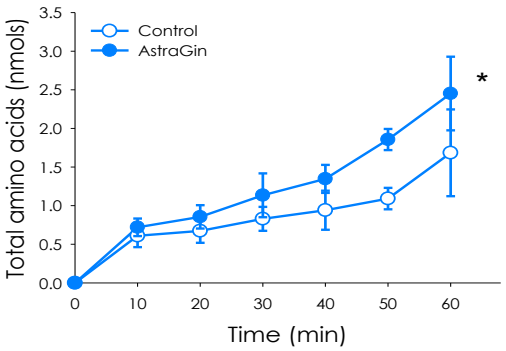

Figure 7. Effect of AstraGin® on total amino acids absorption in Caco-2 cells between 0-60 min after 24h treatment. ECO protein isolate are digested by trypsin, pepsin, pancreatin and AstraZyme™ for 2h, and then added into Caco-2 for amino acids study.

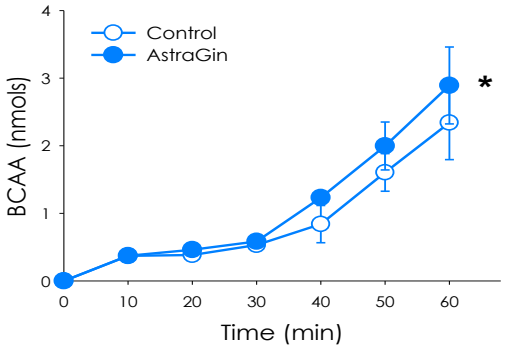

Figure 8. Effect of AstraGin® on BCAA absorption in Caco-2 cells between 0-60 min after 24h treatment. ECO protein isolate are digested by three digestive enzymes, trypsin pepsin and pancreatin for 2h, and then added into Caco-2 for BCAA study.

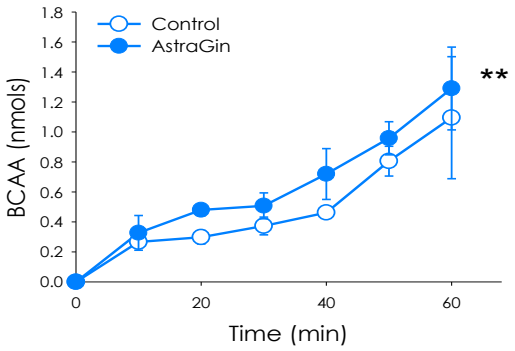

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Figure 9. Effect of AstraGin® on BCAA absorption in Caco-2 cells between 0-60 min after 24h treatment. ECO protein isolate are digested by AstraZyme™ for 2h, and then added into Caco-2 for BCAA study.

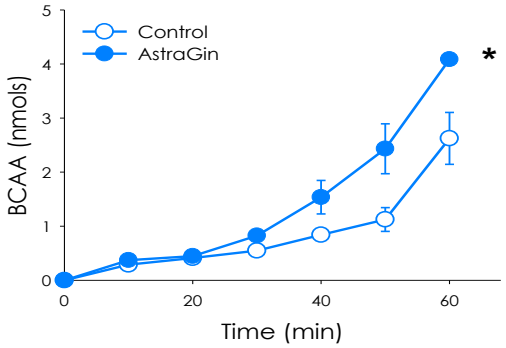

Figure 10. Effect of AstraGin® on total amino acids absorption in Caco-2 cells between 0-60 min after 24h treatment. ECO protein isolate are digested by trypsin, pepsin, pancreatin and AstraZyme™ for 2h, and then added into Caco-2 for amino acids study.