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Study on the absorption and transport of different glutamine dipeptides in small intestine of weaned piglets

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1 SUMMARY

This experiment was conducted to study hydrolysis, absorption and transportation characteristics of Alanyl-glutamine amino acid (Ala-Gln) and Glycyl-glutamine amino acid (Gly-Gln) in small intestine of weaned piglet by detecting the dipeptide and glutamine monomer in culture solution, serosal fluid and tissue of cultured reversal intestine segments *in vitro*. The results showed that, in experiment 1, the content of Ala-Gln and Gly-Gln dipeptides were 9.00±1.57 mmol/L, 8.85±1.78 mmol/L, respectively. In experiment 2, both Ala-Gln and Gly-Gln were detected in the incubated intestinal sac tissue and serosal fluid. The content of Ala-Gln dipeptide transported into the sac was 10.26±0.74 mmol/L, significantly greater than that of Gly-Gln dipeptide, 7.25±1.47 mmol/L (P<0.05). The content of Gln absorbed in Ala&Gln treatment and in Gly&Gln treatment was 7.28±0.54 mmol/L, 4.79±0.54 mmol/L,respectively, with no difference (P>0.05). Absorption content of dipeptides was significantly greater that absorption content of Gln in relative free amino acids treatment. The absorption rate of dipeptides or Gln monomer had the same changing characteristic as the absorption content of dipeptides or Gln monomer. The results suggested that these two dipeptides (Ala-Gln and Gly-Gln) were not totally hydrolyzed in jejunum segments of weaned piglets, and their content gradual decreased. They were absorbed by intestinal cells as intact form, and quickly hydrolyzed. The absorption rate of Ala-Gln was faster than that of Gly-Gln. Dipeptides absorption rate were faster from solutions containing the same amount of amino acids in dipeptide than Gln absorption rate in free form. This study provided the theoretical groundwork for dipeptide nutrition. This suggests that either Ala-Gln or Gly-Gln dipeptides can be used to substitute Gln monomer in weaned piglets feeding.

2 INTRODUCTION

The study of clinical nutrition proved that dipeptids of Ala-Gln and Gly-Gln were highly stable and soluble, when absorbed into body, and they would quickly disintegrate into Gln and the relative amino acids (Stehle *et al*, 1984; Arii *et al*, 1999). The glutamine dipeptides were safe, so they were usually used to take the place of Gln monomer (Albers *et al*, 1989; Klassen *et al*, 2000; Oda *et al*, 2008). The Study on the pig showed that glutamine dipeptides had the same physiological action as Gln monomers. The Animal's intestinal tract has a peptides transport system that transports small peptides into enterocytes (Paulsen & Skuuray, 1994;

Daniel *et al*, 1996; Ogihara *et al*, 1996). There are peptidases in small intestine intestinal brush border and cytoplasm, which hydrolyze dipeptides before transport. Therefore, it has bee difficult to study the absorption of dipeptides. For the past few years, there were lots of researches about absorption of dipeptides, and with much progress (Adibi 1971; Webb *et al*, 1993; Matthews *et al*, 1979; 1995; Gilbert *et al*, 2008). But, there are few reports about absorption of dipeptides of weaned piglets. For this reason, this study dealt with the hydrolysis characteristics of Ala-Gln and Gly-Gln in small intestine of weaned piglet by culturing reversal intestine segments *in vitro*. On this basis, the transport of Ala-Gln and Gly-Gln dipeptides was studied by culturing everted sacs of pig small intestines *in vitro*.

3 MATERIALS AND METHODS

3.1 Materials: Surgical instruments were used in the whole experiment to conduct the surgical procedures, and the 50-ml flasks were sued to incubate the intestinal everted sacs. Homoeothermic incubator was used to provide a befitting temperature for incubating the intestinal sacs. Glass homogenizer was used to homogenize the intestinal tissues, and the hypothermia super centrifuge was used to centrifuge the solution samples. Spectrophotometer (UV-1100) was used to detect the content of glutamine monomer, while the High Performance Liquid Chromatograph (Agilent , HP-1100) was used to detect the content of glutamine dipeptides.

Composition of Krebs solution was in grams per liter: NaCl, 8.0; KCl, 0.3; CaCl2, 0.2; MgCl2, 0.2; glucose, 7.0; HEPES, 6.0, pH7.2 (Hill et al, 1987). The 20 mM Ala-Gln dipeptide sulution, 20 mM Gly-Gln dipeptide sulution, 20 mM Ala&Gln free amino acids solution, and 20 mM Gly&Gln free amino acids solution were prepared by weighing corresponding weight of dipeptides , amino acids and dissolving in Krebs solution respectively to make their concentration be 20 mM. 0.1% Trifluoroacetic Acid (TFA) aqueous solution was prepared by volume ratio, 1 TFA: 1000 H2O, pH 2.0, while the 0.1%TFA-Methyl Cyanides solution was 1 TFA: 1000 Methyl Cyanides.

3.2 Animals and Experimental Groups: PIC pigs were used throughout. Piglets was weaned at 28 days of age and starved for 12 hr before they were killed. In Experiment 1, this experiment was used to study the hydrolysis of glutaminate dipeptides in the jejunum segments culture solution. Three kinds of incubation solutions were prepared, Krebs solution (Control), second was Krebs solution plus 20 mM Ala-Gln, and the third one was Krebs solution plus 20 mM Gly-Gln. Experiment 2 was designed to investigate the absorption of glutaminate dipeptides in culture solution of everted sac jejunum. There were five incubation solutions, one was Krebs solution (Control), two of them were free amino acids (Treatment 1, 20 mM Ala & Gln; Treatment 2, 20 mM Gly & Gln), and the other two solutions were glutamine dipeptides (Treatment 3, 20 mM Ala-Gln; Treatment 4, 20 mM Gly-Gln).

3.3 Preparation of intestinal sacs: Piglets were anaesthetized by the intravenous injection of chlorpromazine and subsequently killed by exsanguinations. The abdomen was immediately opened and the jejunum was excised and dissected into small segments, each approximately 6 cm long, and everted over a glass rod (Wilson & Wiseman, 1954; Pierce & Smith, 1967; Brown et al, 1968; Smith, 1971; Hill et al, 1987). The everted intestinal segments, approximately 4 cm long were rinsed in ice-cold modified Krebs solution. In experiment 1, the intestinal segment was put in a 50-ml flask containing 40 ml relative Krebs solution, each flask one jejunum segment and there were three segments as a group. Then they were incubated under an atmosphere of 95% oxygen (O2) for 1hr at 37C using a single water bath. In experiment 2, first, ligated one end of the everted sac with a surgical suture, and filled with 1 ml Krebs solution (as serosal fluid) with a injector, and then ligated the other end of the sac. The everted intestinal sacs were then put in a 50-ml flask containing 30 ml relative Krebs solution (hanging in the flask), each flask one sac, and three sacs as a group. The incubation condition was the same as in experiment 1, incubated for 40 min.

3.4 Analytical procedures: In experiment 1, 1 ml incubation solution was taken every 5 min from each incubation flask containing Ala-Gln dipeptide or Gly-Gln dipeptide solutions. Samples were incubated in boiling water for 5 min, and were deproteinized with 6% Perchloric acid, and

centrifuged at 4C, 10000r/m for 15 min. The supernatant was rapidly frozen in ultra deep freeze equipment and stored at -70C for detecting.

In experiment 2, at the end of incubation, culture solutions were taken from each flask containing dipeptides or amino acids solutons, and the sacs were removed out from the flasks. Open one end of the everted sac, and extruded the serosal fluid. And then the tissues were incubated in boiling water for 5 min, cooled, and homogenated by glass homogenizer to prepare tissue homogenate fluid. The following procedure of tissue homogenate fluid, culture solutions and serosal fluid were incubated in boiling water for 5 min, and were deproteinized with 6% Perchloric acid, and centrifuged at 4C, 10000r/m for 15 min. The supernatant was rapidly frozen in ultra deep freeze equipment and stored at -70C for detecting.

3.5 HPLC analysis: All the samples taken in experiment 1 and experiment 2 were analyzed for peptides by HPLC at 220 nm on a Nova-Pak 3.9 mm×150 mmC18 column (Zhao, 1998; Dai, 2002). The mobile phase A was 0.1%TFA aqueous solution (volume ratio, 1:1000, pH 2.0). The mobile phase B was 0.1TFA-Methyl Cyanides solution (volume ratio, 1:1000). The flow rate was 1.0 ml/min. Column temperature was 30C.

3.6 Glutamine detection: The content of glutamine in samples of experiment 2 was detected by spectrophotometric method. The detecting wave length was 630nm.

3.7 Calculations and Statistical Analyses: All results are expressed as means ±standard error of the mean (SE). Regression analysis of dipeptides hydrolysis was conducted by EXCELL. Statistical comparisons were carried out using one-way analysis of variance to compare sets of concentration data.

4 RESULTS

4.1 Hydrolysis of glutamine-containing dipeptides: Both dipeptides of Ala-Gln and Gly-Gln hydrolyzed in the jejunum segments culture solution are seen in Table 1 and their contents gradually decreased (Fig. 1). After incubated for 40 min, the content of Ala-Gln and Gly-Gln were 13.68±1.82 mmol/L and 10.82±2.74 mmol/L. The Gln content was 6.76±0.26 mmol/L and 8.36±0.52 mmol/L,which were roughly equal to the amount of the degradative dipeptides. This suggested that there was dipeptide hydrolysis , but no dipeptide absorption. Besides, Gly-Gln dipeptide, no Ala-Gln dipeptide, was detected in the control solution, and the average content was 1.42 mmol/L.

Table 1: The content of Ala-Gln and Gly-Gln at different time during in vitro incubation with 20 mmol/L Ala-Gln or Gly-Gln initiate concentration (mmol/L)

Time (min)	Ala-Gln	Gly-Gln
0	19.35±2.94	20.02±0.76
5	19.26±1.55	18.30±0.12
10	17.52±2.35	17.11±0.87
15	16.79±2.19	14.84±3.19
20	17.31±1.92	14.24±4.00
25	16.46±1.81	14.20±2.86
30	15.05±1.66	14.04±3.80
35	13.82±1.62	10.61±1.66
40	13.68±1.82	10.82±2.74
45	11.19±0.87	10.60±2.64
50	11.35±1.56	10.55±2.13
55	9.76±1.49	10.34±2.02
60	9.00±1.57	8.85±1.78

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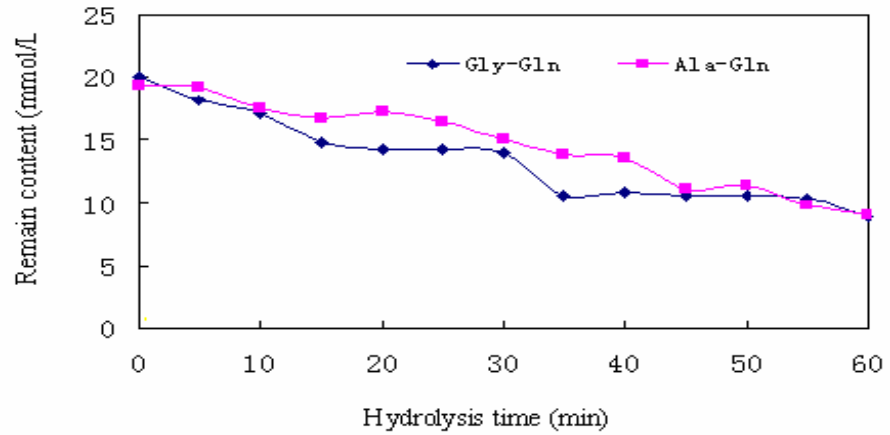

Figure 1: The remainder content of Gly-Gln and Ala-Gln in incubation solution containing 20 mmol/L Gly-Gln or Ala-Gln at different time (min).

4.2 Transportation and absorption of glutamine-containing dipeptides: As shown in table 2, the content of glutamine in culture solution containing Ala-Gln dipeptide solution and Gly-Gln dipeptide was 12.59±0.98 mmol/L and 10.74±1.30 mmol/L, respectively, with no significant difference between the two solutions (P>0.05). This illustrated that some certain dipeptides were hydrolyzed in the intestinal culture solution. The concentration of Ala-Gln and Gly-Gln dipeptide in relative solutions was 1.71±0.71 mmol/L and 7.29±1.59 mmol/L, respectively. Minus the basal content of Gly-Gln dipeptide in the control group, the absorption amount of Ala-Gln and Gly-Gln dipeptide in relative solutions was 10.26±0.74 mmol/L and 0.25±1.47 mmol/L, and the abortion content of Ala-Gln dipeptide was greater than that of Gly-Gln dipeptide (P<0.05). The content of Gln in Ala&Gln and Gly&Gln culture solution was 17.28±0.54 mmol/L, 19.79±0.51 mmol/L, respectively, with no significant difference (P>0.05). This demonstrated that the amount of Gln absorbed in free Ala&Gln and Gly&Gln culture solutions was not much, 7.28±0.57 mmol/L and 4.79 ±0.54mmol/L, respectively. The absorption and transportation content of Ala-Gln and Gly-Gln dipeptide was notably greater than that of Gln in Ala&Gln and Gly&Gln culture solution, respectively (Fig.2). But there were no significant difference between the two free amino acids culture solutions (P>0.05). The absorption and transportation rate of dipeptides and Gln monomer had the same changing tendency in each testing solution (Fig.3). The rate of Ala-Gln was predominately faster than that of Gly-Gln (P<0.05), and both faster than the rate of Gln monomer in relative free amino acids treatment solutions. There was no significant difference between the two free amino acids culture solutions (P>0.05).

The content of dipeptide and Gln monomer in tissue and serosal fluid were detected (table 2). There were basal content of Gly-Gln dipeptide and Gln in tissue and serosal fluid from control group, but no Ala-Gln. The content of Gln in tissue from Ala&Gln treatment was almost equal to that from control treatment, but the content in serosal fluid was significantly greater from Ala&Gln treatment than from control (P<0.05). And the content of Ala-Gln dipeptide in tissue from Ala&Gln treatment was 0.12±0.08 mmol/L. this stated that part of free Ala and Gln absorbed into tissue could be used to synthesize Ala-Gln dipeptide, and part of free Gln absorbed was transported into serosal fluid. The content of Ala-Gln dipeptide and Gln in intestinal tissue from Ala-Gln solution was 0.53±0.05 mmol/L and 10.15±0.68 mmol/L, respectively. They were significantly greater than that from Ala&Gln treated solution (P<0.05). In serosal fluid, there was not any Ala-Gln dipeptide detected, but the Gln content was 7.41±0.96 mmol/L, notably greater than that from Ala&Gln treated solution (P<0.05). The results illustrated that after being transported into the tissue, a part of Ala-Gln dipeptides were kept in the tissue as intact form, a part of them were hydrolyzed into free Ala and Gln, and most of Gln were transported into the serosal fluid. Probably, most of the Ala-Gln dipeptides as intact form were transported into the

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serosal fluid and then rapidly hydrolyzed.

The concentration of Gly-Gln dipeptide in tissue and serosal fluid from Gly&Gln treated solution, control solution and Gly-Gln treated solution were at the same level, but the content of Gln from Gly&Gln treated solution was predominately greater than that from control solution ($P<0.05$). This suggested that most of the Gln absorbed were transported into serosal fluid, only small amount was kept inside the tissue. The content of Gly-Gln dipeptide in serosal fluid from Gly-Gln treated solution was significantly greater than that from Gly&Gln treated solution, but there were no significant difference between the two groups in tissue ($P>0.05$). The result suggested that part of the absorbed Gly-Gln dipeptide were kept inside the tissue, part of them were hydrolyzed into free amino acids, and part of them were transported into serosal fluid as the form of dipeptide or free amino acids.

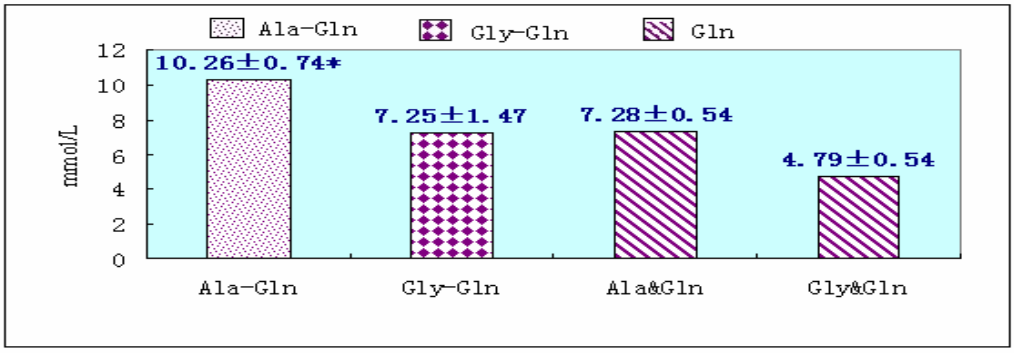

Figure 2: Ala-Gln, Gly-Gln and free Gln absorption content in jejunum (mean±SE)

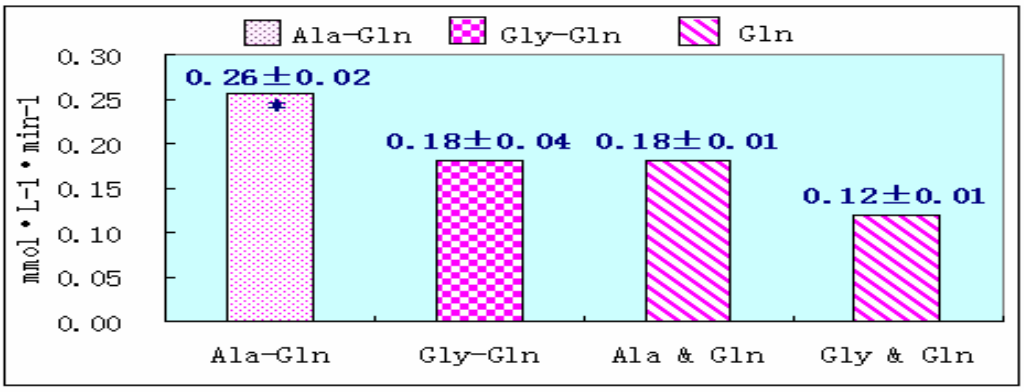

Figure 3: Ala-Gln, Gly-Gln and free Gln absorption rates in jejunum (mean±SE)

5 DISCUSSION

5.1 Hydrolysis of glutamine-containing dipeptides: The first precondition to study dipeptides absorption is the dipeptides' anti-hydrolysis ability. The evidence of Adibi&Kim (1981)shows that the peptidases exist either in the cytoplasm or on the surface of the epithelial cells. These findings suggest that peptide hydrolysis may occur primarily inside the mucosal cell rather than in the intestinal lumen, a possibility stated by Newey and Smyth (1959b). But the presence of peptidases on the enterocyte brush border, as well as in the cytosol and the submucosal compartment, makes the site of hydrolysis for any particular peptide uncertain. It is generally accepted that the small peptides become hydrolysed, their breakdown occurring to a limited extent within the intestinal lumen, by the action of peptidases secreted from the pancreas and peptidases that are present in desquamated mucosal cells (Wiley & Sons, 1987). Most of the small peptides formed in the intestine are hydrolysed by peptidases associated with outer (digestive-absorptive) surface, the brush border of the epithelial cells, rather than by peptidases acting inside the mucosal cells into which the peptides are absorbed.

Gardner and Plumb (1979) and silk and kim (1976) report that peptidases are rapidly released

from *in vitro* preparations of intestine unless the preparation is set up while the animals are under anaesthesia. This means that the dipeptides will rapidly be hydrolyzed by the peptidases released from intestine in vitro. Thus, many researchers can't detect depeptides *in vitro* (Zhao, 1998). Previous reports have also suggested that Gly-Gly dipeptide is hydrolysed by peptidases released from the intestine in everted sacs *in vitro* in 5 min, and the Gly-Leu dipeptide in 25 min (Liu *et al,* 2002; Zhang, 2004). L-alanyl-D-phenylalanine is rapidly hydrolysed within the mucosa (Lister *et al*, 1995). But dipeptides with an N-terminal D-amino acid are relatively resistant to hydrolysis by cytosolic peptidases. Lister *et al.* (1995) shows that these dipeptides are transported intact across the epithelial layer. Some small oligopeptides, such as glycyl-proline, are also relatively resistant to hydrolysis (Cheeseman and Johnston, 1982). Gly-Leu and His-Leu are not hydrolyzed in small intestinal brush border membrane vesicles of piglets (Dai, 2005). All these findings suggest that different dipeptides have different ability to resistant hydrolysis, or that the kinds of peptidases released from the intestine are different with different animals. After 60 min in Exp.1, the amount of Ala-Gln and Gly-Gln dipeptide are 46.5% and 44.3% of initiate content, which reveals that both the dipeptides are not totally hydrolyzed, and that they are hydrolysis-resistant to some extent. The fact that the mucosal content of hydrolysis-resistant peptides measured at the end of the Experiment 1 also gives the evidence that some dipeptides are hydrolysis-resistant.

Both Ala-Gln and Gly-Gln dipeptide are synthesized dipeptides. They are highly soluble and stable at room temperature. Arii *et al.* (1999) points out that Ala-Gln dipeptide is the most stable at pH 6.0, and it can be preserved for 5.3 years and 7.1 months at the temperature of 25℃ and 40℃ respectively, and 90 percent of dipeptides don't degrade. The stability of glutamine-containing dipeptides is Gly-Gln > Ala-Gln > Leu-Gln > Val-Gln > Ile-Gln. But this study's result that the hydrolyzed amount of Ala-Gln is lower than Gly-Gln, suggests that Gly-Gln is more easily hydrolyzed by peptidases released from the intestinal cells of weaned piglet than Ala-Gln.

5.2 Transportation and absorption of glutamine-containing dipeptides: It is generally believed that most di- and tripeptides are absorbed intact from the lumen by the mucosa of the small intestine even though it is normally only the constituent amino acids which appear in the vascular system (Gardner, 1988; Matthews, 1991; Meredith and Boyd, 1995). It is important to recognize that the transport of peptides across the epithelial layer requires three distinct steps: uptake by the brush border, transfer to the basolateral pole of the enterocyte and exit across the basolateral membrane. In this study, isolated sacs of intestine were used, because this enables us to look at transmural transfer from lumen to serosa, as well as providing us with the amount of dipeptides absorbed and the form of dipeptides after absorption. The fact that the content of hydrolysis-resistant peptides were measured at the end of the Exp.2 in the serosal fluid and tissue provides evidence on the absorption of dipeptides as intact form.

The data of this paper gives a clear picture of the fate of dipeptides of glutaminate plus glycine or alanine contained in the incubation solution of sacs of the weaned piglet small intestine. Experiments in vitro on the absorption of peptides have shown that small quantities of glycylglycine can penetrate through the intestine (Newey & Smyth, 1959a; Wiggans & Johnston, 1959). The two synthesised dipeptides are both completely absorbed, and then hydrolysed within the mucosa after absorption from the loops. This study results with these two dipeptides are in agreement with the data obtained for the transport of D-leucyl-D-leucine by Boyd and Ward (1982) in *Necturus* intestine and by Lister et al. (1995) in rat small intestine. The amount of Ala-Gln (10.26±0.74 mmol/L) absorbed was higher than that of Gly-Gln (7.25±1.47 mmol/L). The dipeptide with L-alanine in the N-terminal position gives the highest rate of Ala-Gln transfer which supports the view that a large lipophilic side chain at the N-terminus of the peptide enhances transport (Matthews, 1991).

There are two possible passing ways of the dipeptides absorbed into the epithelial cells, one is by passing into the portal vein and ultimately into the liver, but with a few content, another one is to be hydrolyzed by peptidases acting inside the mucosal cells into which the peptides are absorbed. Lister *et al.* (1995) points out that L-Alanyl-L-phenylalanine, L-Alanyl-D-phenylalanine, L-Phenylalanyl-L-alanine and L-Phenylalanyl-D-alanine are completely hydrolysed following uptake from the lumen, butt

they are confident that they are absorbed prior to hydrolysis because the perfusions are single-pass experiments and there is no detectable free phenylalanine in the luminal effluent. The fact that there was no evidence for the presence of Ala-Gln in the mucosa when the intestinal sacs were incubated in the Ala-Gln solution, means that it was rapidly hydrolysed within the mucosa cells. Only modest amount of Gly-Gln was detected in serosal fluid and tissue from Gly-Gln solution incubated sacs and the amount of glutaminate in the related fluids was higher than that from Gly&Gln incubated sacs. The fact that intact Gly-Gln in the serosal fluid was detected is a consequence of a rapid rate of absorption from the sac together with a more modest inhibition of hydrolysis. However, the results reported by Han (2003) who considers that all the dipeptides absorbed are totally hydrolysed into free amino acids, are at variance with ours. This study's data makes it clear that the absorption amount of glutaminate from dipeptides are higher than that from free amino acids. This means that amino acid absorption rates are significantly greater from solutions containing the same amount of amino acids in dipeptide than in free form.

7 CONCLUSION

The results point to four general conclusions about intestinal peptide absorption and transport which should now be tested with other peptides. First, both Ala-Gln and Gly-Gln show some hydrolysis-resistant ability to peptidases released from intestinal sacs of weaned piglet. Second, both glutamine-containing dipeptides are absorbed as intact form, rapidly hydrolysed into free amino acids after absorption and the absorption content are 10.26±0.74 mmol/L and 7.25±1.47 mmol/L, respectively. Third, the glutaminate absorption content are 7.25±0.54 mmol/L and 4.79±.54 mmol/L from free Ala & Gln and free Gly & Gln solutions, respectively. Finally, the absorption rate of dipeptides are greater than that of free amino acids. This study provided the theoretical groundwork for dipeptide nutrition. This suggests that either Ala-Gln or Gly-Gln dipeptides can be used to substitute Gln monomer in weaned piglets feeding.

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Table 2: The content of Gly-Gln, Ala-Gln dipeptide and Gln monomer in different fluid (mmol/L)

		Control	Ala & Gln	Ala-Gln	Gly & Gln	Gly-Gln
Ala-Gln dipeptide	Serosal fluid	0^{a}	0^{a}	0^{a}		
	Incubation solution (mucous membrane)	0^{a}	0^{a}	1.71 ± 0.71^{b}		
	Tissue homogenate fluid	0^{a}	0.12 ± 0.08^{a}	0.53 ± 0.05^{b}		
Gly-Gln dipeptide	Serosal fluid	1.33 ± 0.06^{a}			1.47 ± 0.28^{a}	1.6 ± 0.22^{a}
	Incubation solution (mucous membrane)	1.34 ± 0.06^{a}			1.32 ± 0.08^{a}	7.29 ± 1.59^{b}
	Tissue homogenate fluid	6.31 ± 0.42^{a}			6.71 ± 0.08^{a}	7.23 ± 0.45^{a}
Gln monomer	Serosal fluid	0.94 ± 0.17^{a}	4.80 ± 0.64^{b}	7.41 ± 0.96^{c}	4.94 ± 0.23^{b}	8.75 ± 0.78^{d}
	Incubation solution (mucous membrane)	4.56 ± 0.06^{a}	17.28 ± 0.54^{c}	12.59 ± 0.98^{b}	19.79 ± 0.51^{c}	10.74 ± 1.30^{b}
	Tissue homogenate fluid	5.62 ± 0.36^{a}	5.82 ± 0.47^{a}	10.15 ± 0.68^{c}	7.83 ± 0.49^{b}	7.70 ± 0.64^{b}

*Each value represents the mean ± SEM determined in three subjects. In the same line, values with different lower case letter superscripts mean significant difference ($P<0.05$). Values with same letter superscripts mean no significant difference ($P > 0.05$)