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Usage of Enzyme Substrate to Protect the Activities of Cellulase, Protease and α-Amylase in Simulations of Monogastric Animal and Avian Sequential Total Tract Digestion

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ABSTRACT : Cellulase from *Aspergillus niger*, (α-amylase from Bacillus sp. and protease from Bacillus globigii were used as enzyme sources in this study to examine how their respective substrates protect them in two kinds of simulated gastrointestinal tract digesting processes. Avian total digest tract simulation test showed that filter paper, Avicel and cellulose resulted in 7.7, 6.4 and 7.4 times more activity than of unprotected cellulose, respectively. Protease with addition of casein, gelatin or soybean protein showed no significant protection response. Starch protected amylase to be 2.5 times activity of the unprotected one. Monogastric animal total tract digestion simulation test showed that filter paper, Avicel and cellulose resulted in 5.9, 9.0 and 8.8 times activity of unprotected cellulase, respectively. Casein, gelatin and soybean protein resulted in 1.2, 1.3 and 2.0 times activity of unprotected protease, respectively. Starch did not protect amylase activity in monogastric animal total tract simulation. Protection of mixed enzymes by substrates in two animal total tract simulation tests showed that filter paper in combination with soybean protein resulted in 1.5 times activity of unprotected cellulose, but all substrates tested showed no significant protection effect to protease. Soybean protein and starch added at the same time protected the amylase activity to be two times of the unprotected one. Test of non-purified substrate protection in two animal total digest tract simulation showed that cellulase activity increased as BSA (bovine serum albumin) concentration increased, with the highest activity to be 1.3 times of unprotected enzyme. However, BSA showed no significant protection effect to protease. Amylase activity increased to 1.5 times as BSA added more than 1.5% (w/v). Cellulase activity increased to 1.5 times as soybean hull was added higher than 1.5%. Amylase had a significant protection response only when soybean hull added up to 2%. Protease activity was not protected by soybean hull to any significant extent. (**Key Words :** Enzyme Substrate, Activity Protection, Cellulase, Protease, Amylase)

INTRODUCTION

Many polysaccharides can form viscous gel-like structure in the small intestine which will trap nutrients and hinder the action of the animal's digestive enzymes. Cellulases and xylanases added to the diet can break down these gels and release the trapped nutrients (Pettersson and Aman, 1989). However, positive responses were not achieved all the time for fibrolytic enzymes' application in pigs or broilers (Kim et al., 2004; Qiao et al., 2005). Some cellulases, expressed by bacteria and fungi, are glycosylated by post-translational modification which often protects enzymes from inactivation by heat or protease (Olsen and Thomsen, 1991). The stability of cellulase can also be improved by modification with synthetic copolymers over a wide range of pH (Jin, 1996). Fontes et al. (1995) indicated that labile cellulases were resistant to proteolytic attack in the presence of their appropriate substrate. Many commercial feed enzyme additives are mixtures of protease, cellulase and amylase. In this case, protease may attack other enzymes and decrease their efficiency. To be fully functional in the digestive tract, exogenous enzymes should be resistant to attack of protease in the small intestine, and able to exhibit catalytic activity in the pH range 6 to 8. To maintain the activity of exogenous enzymes, substrate addition seems to be a convenient and cheap method to protect enzymes. In this study, purified microbial protease, cellulase and amylase were used alone or mixed for various tests of substrates' protection ability. Common protease substrates (casein, gelatin or soybean protein), cellulase

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Table 1. List and abbreviations of substrates used for protection ability tests for enzyme mixture

Cellulase and protease mixture protection test	
Cellulose substrate	Protein substrate
Cellulose (C)	Casein (C)
Avicel (A)	Gelatin (G)
Filter paper (F)	Soybean protein (S)
No cellulase substrate (E)	No protease substrate (N)
Amylase and protease mixture protection test	
Starch substrate	Protein substrate
Starch (S)	Casein (C)
No amylase substrate (E)	Gelatin (G)
	Soybean protein (S)
	No protease substrate (N)

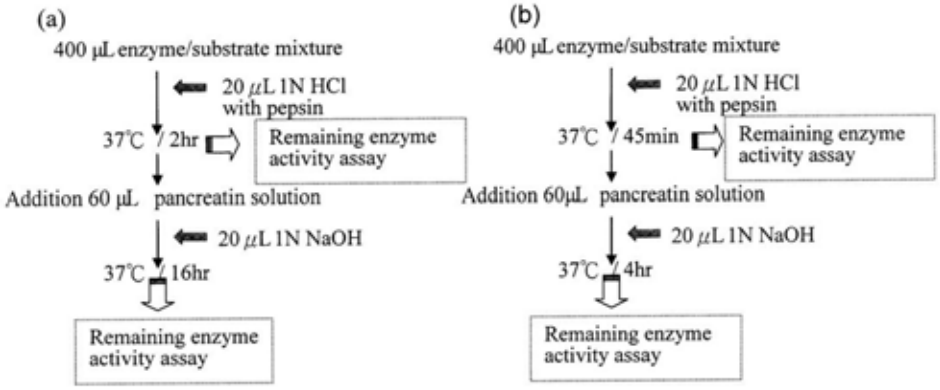

Figure 1. *In vitro* digestion simulation procedures for substrate protection test. (a) Simulation of monogastric animal digestion sequence. (b) Simulation of avian digestion sequence.

substrates (Avicel, cellulose or filter paper) and amylase substrate (corn starch) were tested for their protection ability on their respective enzymes. Finally, the non-purified substrate BSA and soybean hull were tested for their protection ability toward all three kinds of enzymes. The objective of the study was to find a better way to supply mixed enzyme feed additives and exploit low cost enzyme protection materials for various enzymes.

MATERIAL AND METHOD

Enzyme sources

All enzymes and reagents used in this study were obtained from Sigma Co. (St. Louis, MO, USA). Cellulase (EC 3.2.1.4) was from *Aspergillus niger* (Sigma C-1184), protease (EC 3.4.21.14) was from *Bacillus globigii* (Sigma P-5459) and α-amylase (EC 3.2.1.1) was from *Bacillus sp.* (Sigma A-6814).

Enzyme activity in different pH

To study the individual and mixed enzymes activity under different pH conditions, glycine-HCl buffer (pH 3), sodium-phosphate buffer (pH 7) and HEPES buffer (pH 8) were used. Cellulase (3 mg/ml, 3.5 units/ml), protease (0.0438 mg/ml, 0.45 units/ml), α-amylase (5 mg/ml, 250 units/ml) or mixtures of all three enzymes were dissolved in different pH buffers and incubated at room temperature for 10 minutes, followed by immersion in ice to stop the reaction and enable assay of residual activities..

Enzyme activity tests in simulation of stomach and small intestine conditions

To simulate the stomach conditions, pepsin solution was prepared by dissolving porcine pepsin (EC 3.4.23.1, Sigma P-7000) in 50 mM glycine-HCl buffer (pH 2 or 3) to a final concentration of 75 units/ml. Trypsin and chymotrypsin solution for simulating the small intestinal conditions were prepared by dissolving trypsin (Sigma, T-0303) and chymotrypsin (Sigma, C-4129) in 50 mM Tris-HCl buffer to a final concentration of 10 mg/ml. The concentrations of cellulase, protease and α-amylase used were the same as in the previous pH test.

Substrate protection ability tests

Casein, soybean protein and gelatin were tested for their protective efficacy on protease. Cellulose, Avicel PH-101 and filter paper (Whatman No.1) were tested for their protective efficacy on cellulase. Corn starch, the common

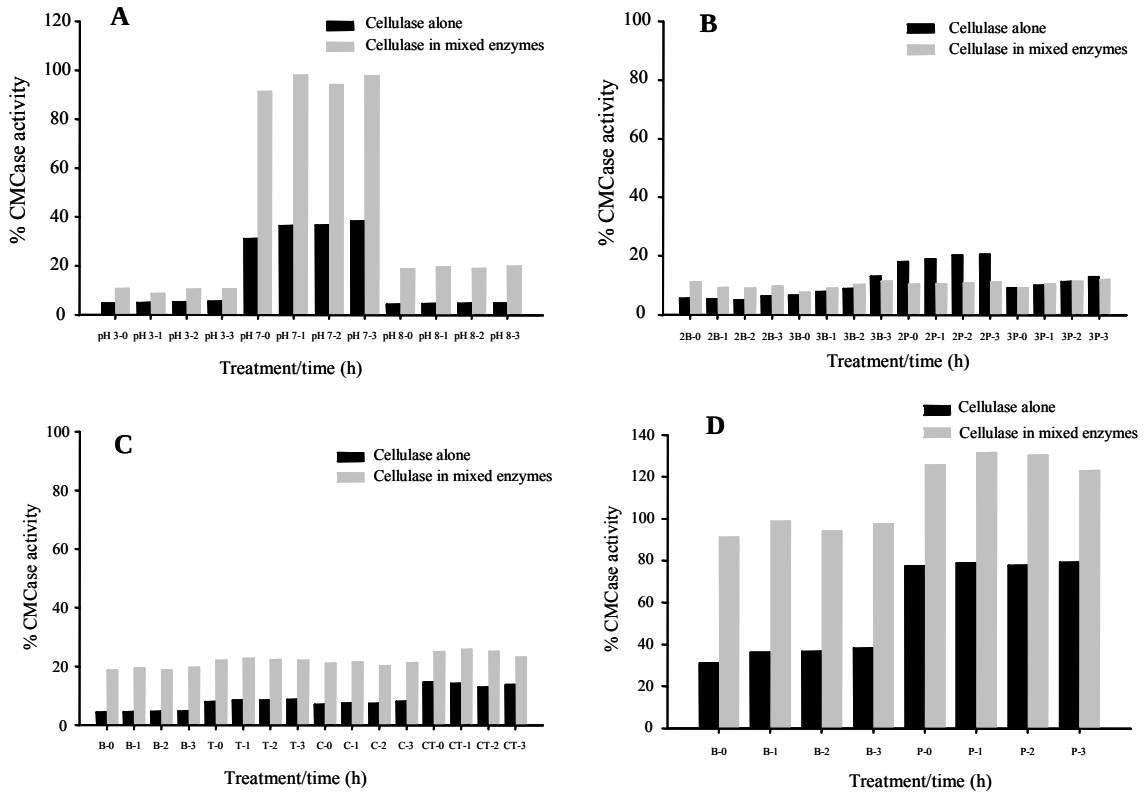

Figure 2. The effects of pH and enzyme in simulation of gastrointestinal tract digestion on cellulase activity (cellulase activity with buffer only was set as 100%). (A) pH buffer only. (B) B: buffer (pH 2 or 3), P: buffer+pepsin (75 U/ml). (C) B: buffer (pH 8), T: buffer+trypsin (10 mg/ml), C: buffer+chymotrypsin (10 mg/ml), CT: buffer+trypsin+chymotrypsin. (D) B: buffer (pH 6.8), P: buffer+pancreatin (10 mg/ml).

starch source was used to test its protective efficacy on α-amylase. Cellulase, protease and α-amylase were diluted five times to final concentrations of 0.6 mg/ml, 0.00876 mg/ml and 1 mg/ml, respectively. All tested substrates were dissolved in water before mixing with enzyme solution. Enzymes were dissolved in 100 mM sodium phosphate buffer (pH 6.8) and mixed with the substrate solution at a 1:1 ratio. Lists and abbreviations of substrates used in the protection ability test for cellulase-protease or amylase-protease mixtures are shown in Table 1. To determine the protection ability for individual or mixed enzymes, two kinds of *in vitro* digestion simulation procedures were employed (Figure 1). Remaining activities of exogenous enzymes in both stomach and sequential total tract simulation condition were analyzed to determine the protection effect from different substrates.

Non-purified substrate protection ability tests

Soybean hull and BSA were individually mixed with three kinds of enzymes to test their protection ability for cellulase, protease and α-amylase. Soybean hull was ground through the 30 mesh screen before use. Enzyme concentration and *in vitro* test procedures were performed as previously described for substrate protection ability tests.

Enzyme assay methods

Cellulase activity was assayed by using carboxymethyl cellulose (CMC) as substrate. Assays were carried out by adding 100 μl of sample to a tube contain 100 μl of 50 mM sodium phosphate buffer with 1% CMC. The mixture was incubated at 37°C for 20 min, then the reaction was stopped by addition of Somogyi reagent, and reducing sugars were measured by the Nelson- Somogyi method (Wood and Bath, 1988). Alpha-amylase activity was measured by blocked p-nitrophenyl maltoheptaoside (BPNPG7, Sigma N-1519) (McCleary and Sheehan, 1987). Samples (50 μl) were incubated with 50 μl BPNPG7 (4 mM) at 37°C for 1 h. The reaction was terminated and colour developed by addition of 1% (w/v) Trizma base (750 μl, pH>10) and the release of p-nitrophenol was measured by 405 nm absorbance. For assay of protease activity, the azocasein method (Brock et al., 1982) was used. Azocasein solution (0.8%) was prepared by dissolving 8 mg azocasein in 1.0 ml of 100 mM sodium-phosphate buffer (pH 6.8). Azocasein solution (200 μl) was pipetted into a 1.5 ml microcentrifuge tube and 200 μl enzyme sample solution added. The mixture was incubated at 37°C for 20 min and the assay terminated by adding 200 μl of 1.5 M $HClO_4$ afterwards. The contents were mixed thoroughly and allowed to stand in ice for 1 h

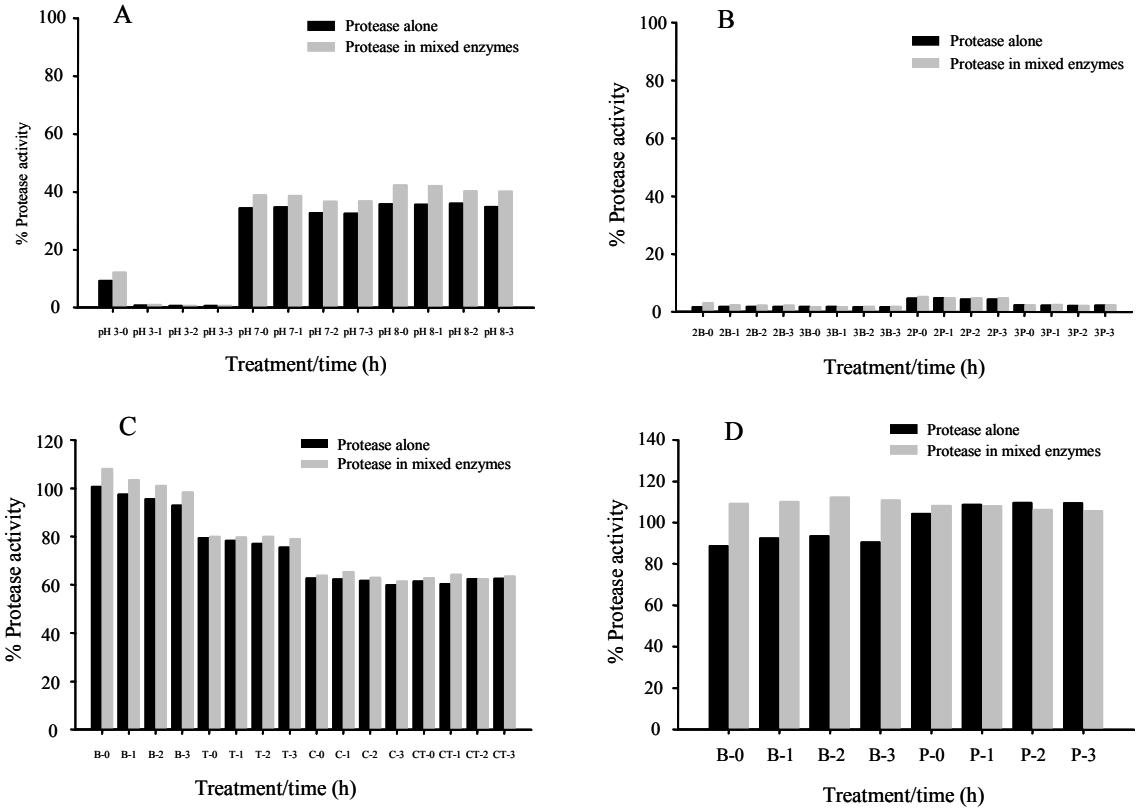

Figure 3. The effects of pH and enzyme in simulation of gastrointestinal tract digestion on protease activity (protease activity with buffer only was set as 100%). (A) pH buffer only. (B) B: buffer (pH 2 or 3), P: buffer+pepsin (75 U/ml). (C) B: buffer (pH 8), T: buffer+trypsin (10 mg/ml), C: buffer+chymotrypsin (10 mg/ml), CT: buffer+trypsin+chymotrypsin. (D) B: buffer (pH 6.8), P: buffer+pancreatin (10 mg/ml).

to ensure complete precipitation of the remaining azocasein. The samples were centrifuged at 15,000×g for 10 min, 100 μl supernatant fluid transferred to a microplate and an equal volume of 1 N NaOH added. Absorbance was determined at 450 nm.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) procedures using the SAS system for Windows (SAS 8.0, SAS Institute). Differences between treatment means were determined using Duncan's test with significance of difference set at $p<0.05$.

RESULTS AND DISCUSSION

The effects of pH and digestion enzyme

Cellulase : Cellulase activity in different pH buffers with or without pepsin or pancreatic enzyme is shown in Figure 2. Residual cellulase activity in mixed enzymes was higher than cellulase applied alone in all tested conditions. Cellulase had high residual activity at neutral pH, but activity decreased toward basic conditions. The cellulase activity decreased in low pH or pepsin treatment (Figure 2A and 2B), but trypsin and chymotrypsin treatment had no significant effect on cellulase (Figure 2C). Cellulase in mixed enzyme tests was more stable in trypsin and chymotrypsin treatment or pancreatin treatment than cellulase applied alone (Figure 2C and 2D).

The cellulase used in our study was from *Aspergillus niger* with an optimal pH at 5.0. In the present study, cellulase of *Aspergillus niger* was more stable at pH 7.0 than pH 3.0. Cellulase activity in individual and mixed enzyme tests increased immediately after pancreatin treatment, but longer treatment time did not enhance the activity further. The phenomenon of increasing cellulase activity may be due to pancreatin's interference with the color presentation of DNS reagent.

Protease : Activities of protease alone or in mixed enzymes were all significantly depressed by low pH (pH 2 or 3) and pepsin treatment (Figure 3A and 3B), with only 10% activity left in comparison to neutral pH conditions. Exogenous protease was inactivated by trypsin and chymotrypsin (Figure 3C), whereas pancreatin treatment had no significant effect on protease, and retained >85% residual protease activity (Figure 3D).

The optimal pH of protease and amylase in this study was 7.5 and 6.9, respectively. This suggested that protease and amylase were more vulnerable at low pH than cellulase

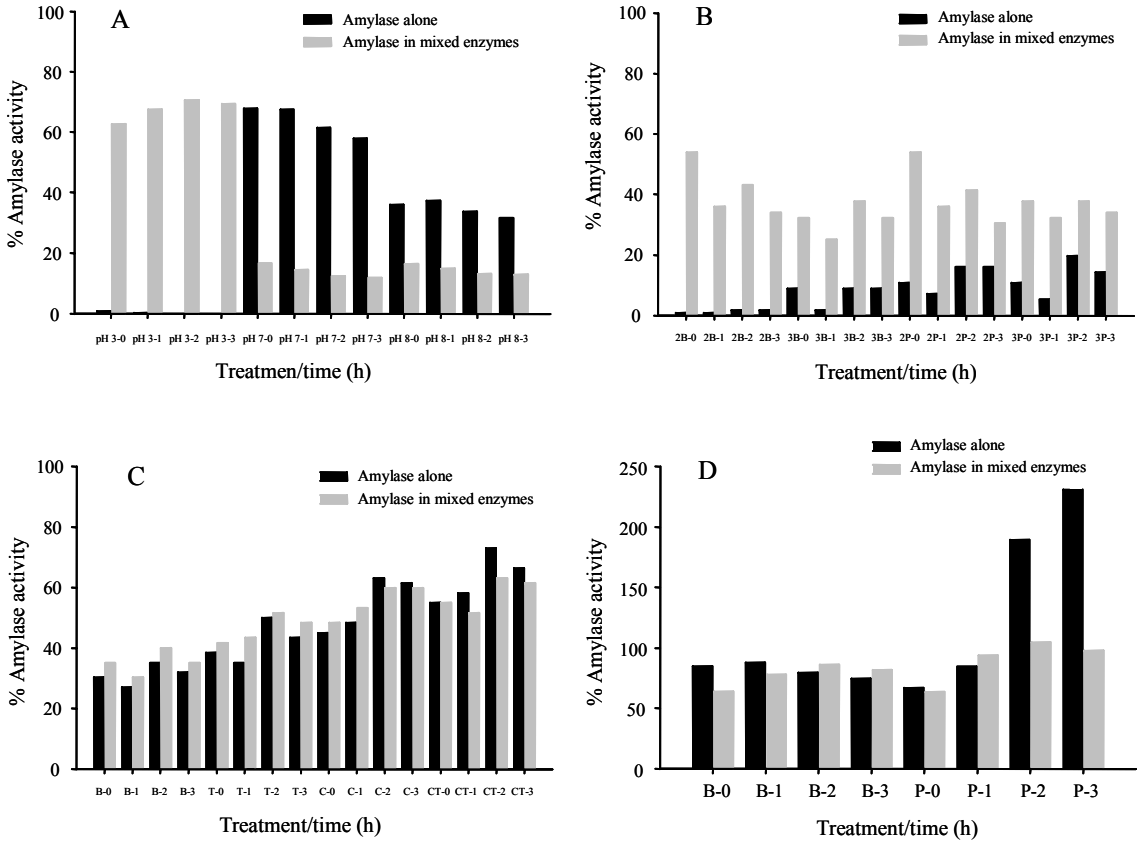

Figure 4. The effects of pH and enzyme in simulation of gastrointestinal tract digestion on amylase activity (amylase activity with buffer only was set as 100%). (A) pH buffer only. (B) B: buffer (pH 2 or 3), P: buffer+pepsin (75 U/ml). (C) B: buffer (pH 8), T: buffer+trypsin (10 mg/ml), C: buffer+chymotrypsin (10 mg/ml), CT: buffer+trypsin+chymotrypsin. (D) B: buffer (pH 6.8), P: buffer+pancreatin (10 mg/ml).

in this study. The protease activity was repressed by low pH condition, but activity recovered after returning to neutral pH.

Amylase : The amylase in mixed enzymes was more stable than amylase alone at low pH or pepsin treatment (Figure 4A and 4B), but amylase activity in mixed enzymes dropped to 20% of its original activity at neutral pH (Figure 4A). This may be related to the higher protease activity in mixed enzymes at pH 7 (Figure 3A). The amylase activity increased after trypsin, chymotrypsin and pancreatin treatment (Figure 4C and 4D). After pancreatin treatment, amylase activity was higher for amylase alone than for mixed enzymes. This may be due to the combined action of protease from mixed enzymes and pancreatin.

Comparing the activity of mixed enzymes with enzyme alone implied that α-amylase in mixed enzymes was more stable in low pH or pepsin treatment than amylase alone. It is possible that enzymes in mixtures have less opportunity to be attacked by pepsin than when applied alone.

Substrate protection ability tests for individual enzyme

Cellulase : All kinds of cellulase substrates can protect cellulase activity in simulations of stomach and total tract digestion (Figure 5A and 5B). In the simulation of total tract digestion, cellulose and Avicel had better protective ability on cellulase (Figure 5B). Both avian and monogastric animal digestive simulation procedures showed that cellulase activity was well protected by its substrates. Residual activities of cellulase with substrate addition were five times higher than without substrate protection.

It is possible that the substrate protected the enzyme activity through occupying the active site of the enzyme and preventing the gastrointestinal tract's enzyme attack. Alternatively, the binding of the substrate to enzyme may cause a conformational change to a much tighter tertiary structure that is more resistant to proteolysis. Addition of 0.2% β-glucan was shown to significantly decrease the sensitivity of protease-labile cellulase or xylanase and increase the residual activity about 20% (Fontes et al., 1995). Many kinds of cellulase have serine-rich sequences which are very sensitive to protease if they are not protected in any way (Gilkes et al., 1991).

Protease : Protease was significantly more resistant to acid and pepsin attack in the presence of soybean protein (Figure 6). When simulating total tract digestion of the monogastric animal, protection was greater with casein addition (Figure 6B), which maintained residual protease

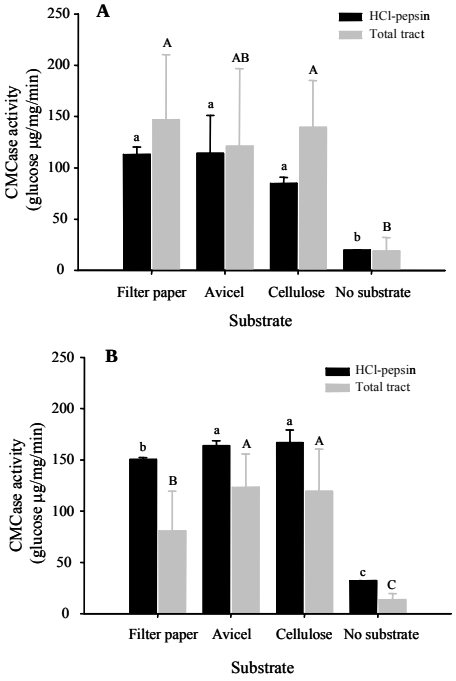

Figure 5. The protection effects of substrates on protection of cellulase activity. (A) Simulation of avian digestion sequence. (B) Simulation of monogastric animal digestion sequence. Superscripts a, b and c denote different in stomach digestion simulation ($p<0.05$). Superscripts A, B and C denote different in total tract digestion simulation ($p<0.05$).

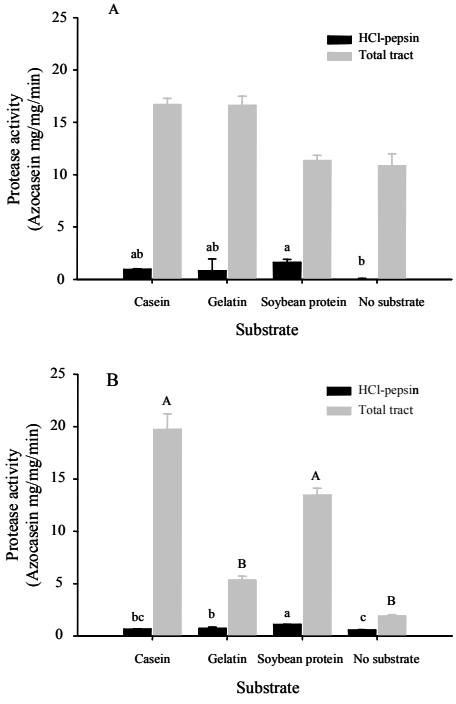

Figure 6. The protection effects of substrates on protection of protease activity. (A) Simulation of avian digestion sequence. (B) Simulation of monogastric animal digestion sequence. Superscripts a, b and c denote different in stomach digestion simulation ($p<0.05$). Superscripts A and B denote different in total tract digestion simulation ($p<0.05$).

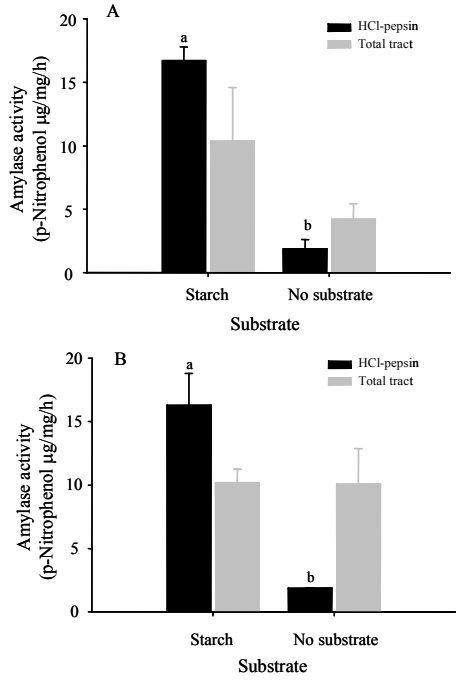

Figure 7. The protections effects of substrates on protection.of amylase activity (A) Simulation of avian digestion sequence. (B) Simulation of monogastric animal digestion sequence. Superscripts a and b denote different in stomach digestion

activity about eight times greater than that without substrate protection. Gelatin addition had less protective ability after an extended digestion procedure than other substrates.

Amylase : Starch addition protected amylase activity insimulation of stomach digestion, but had no protective effect in simulation of total tract digestion conditions (Figure 7A and 7B). The pancreatin used in this study contained amylases which may affect the outcome of total amylase activity in the digestion simulation test.

Substrate protection ability test for mixed enzymes

The arrangement of enzyme mixtures and their substrate combinations is shown in Table 1. Two kinds of enzyme mixtures, (1) cellulase and protease and (2) amylase and protease, were tested.

Cellulase and protease : All cellulose substrates could protect the cellulase activity in stomach digestion simulation as shown in Table 2. The combination of Avicel and soybean protein (AS) had higher protection ability than others, the residual cellulase activity was four times higher than that without addition of any substrate (EN). Compared with enzyme applied alone without substrate, soybean protein addition increased the residual activity of protease about two times in simulation of stomach digestion for both animal modules. Total tract digestion simulation with both animal modules (Table 2) showed that filter paper with soybean protein protected cellulase activity appreciably and maintained 1.5 times more residual cellulase activity. In

Table 2. The protective effects of cellulose substrates and protease substrates in simulations of monogastric animal and avian digestive tracts

	Monogastric animal				Avian			
	Cellulase (glucose μg/mg/min)		Protease (azocasein mg/mg/min)		Cellulase (glucose μg/mg/min)		Protease (azocasein mg/mg/min)	
	Stomach	Total tract	Stomach	Total tract	Stomach	Total tract	Stomach	Total tract
AC	183.747^{abc}	435.658^{abc}	1.268^{ab}	31.219^{bc}	159.071^{ab}	413.435^{a}	2.523^{a}	36.004^{cdef}
AG	183.293^{abc}	376.155^{cdef}	0.990^{abcd}	31.556^{bc}	173.866^{ab}	350.934^{bcd}	1.569^{abc}	36.810^{abcde}
AS	211.724^{a}	460.617^{a}	1.245^{ab}	30.389^{bc}	199.312^{a}	381.500^{abc}	2.111^{ab}	34.923^{fg}
AN	181.438^{abc}	383.343^{bcde}	0.332^{f}	30.362^{c}	155.760^{ab}	339.038^{bcd}	1.317^{bc}	35.849^{def}
CC	152.671^{bc}	369.626^{cdefg}	1.123^{abc}	31.401^{abc}	173.760^{ab}	364.506^{abcd}	2.147^{ab}	36.225^{bcdef}
CG	167.820^{abc}	325.499^{defgh}	0.841^{cde}	31.174^{bc}	160.924^{ab}	342.271^{bcd}	1.895^{ab}	34.737^{fg}
CS	199.080^{ab}	391.952^{bcd}	1.213^{abc}	30.661^{bc}	188.046^{ab}	349.093^{bcd}	1.640^{abc}	34.332^{g}
CN	156.603^{abc}	360.069^{defg}	0.598^{ef}	30.118^{c}	152.009^{ab}	343.499^{bcd}	1.244^{bc}	36.463^{abcde}
FC	159.696^{abc}	304.910^{gh}	1.077^{bc}	32.949^{a}	156.209^{ab}	315.954^{def}	2.468^{a}	37.816^{a}
FG	138.593^{cd}	371.285^{cdefg}	0.844^{cde}	32.161^{b}	148.754^{ab}	332.995^{bcde}	1.920^{ab}	37.803^{a}
FS	182.579^{abc}	447.532^{ab}	1.368^{a}	30.771^{bc}	177.122^{ab}	392.948^{ab}	1.720^{ab}	35.486^{efg}
FN	141.021^{cd}	320.392^{efgh}	0.368^{f}	31.115^{abc}	141.974^{b}	324.640^{cde}	1.128^{bc}	37.261^{abcd}
EC	65.138^{e}	276.081^{h}	1.286^{ab}	31.160^{bc}	58.832^{c}	262.563^{fg}	2.050^{ab}	37.603^{ab}
EG	69.275^{e}	264.312^{h}	0.923^{cde}	31.384^{abc}	63.940^{c}	247.174^{g}	1.570^{abc}	37.508^{abc}
ES	98.345^{e}	306.938^{fgh}	1.335^{a}	30.396^{bc}	87.746^{c}	278.661^{efg}	1.838^{ab}	35.297^{efg}
EN	52.753^{e}	267.471^{h}	0.610^{ef}	30.744^{bc}	50.478^{c}	233.861^{g}	0.690^{c}	36.659^{abcde}
SEM	44.202	12.768	0.183	0.104	37.753	13.430	0.847	0.054

[a, b, c, d, e, f, g, h] Vaules in the same column without the same superscripts are significantly different ($p<0.05$, n = 8).

Table 3. The protective effects of starch substrates and protease substrates in simulations of monogastric animal and avian digestive tracts

	Monogastric animal				Avian			
	Protease (azocasein mg/mg/min)		Amylase (p-nitrophenol μg/mg/h)		Protease (azocasein mg/mg/min)		Amylase (p-nitrophenol μg/mg/h)	
	Stomach	Total tract	Stomach	Total tract	Stomach	Total tract	Stomach	Total tract
CS	1.770^{bc}	30.054^{ab}	5.058^{ab}	19.726^{a}	1.659^{b}	33.673^{a}	7.015^{cd}	11.239^{cd}
CN	1.798^{abc}	30.608^{a}	3.117^{de}	19.356^{abc}	1.627^{b}	33.042^{abc}	5.270^{cd}	9.730^{d}
ES	1.062^{d}	25.160^{d}	4.256^{bc}	19.808^{a}	0.909^{b}	33.813^{a}	11.329^{bc}	10.750^{cd}
EN	1.202^{d}	26.790^{dc}	2.905^{e}	19.651^{ab}	1.017^{b}	33.545^{ab}	2.221^{d}	9.170^{d}
GS	1.618^{c}	28.130^{bc}	3.922^{cd}	19.471^{abc}	1.694^{b}	33.237^{abc}	11.124^{bc}	13.964^{c}
GN	1.538^{c}	29.996^{ab}	3.342^{de}	20.005^{a}	2.665^{b}	34.149^{a}	5.431^{cd}	9.322^{d}
SS	2.072^{ab}	27.906^{c}	5.597^{a}	18.878^{bc}	1.850^{b}	32.226^{bc}	26.904^{a}	22.885^{a}
SN	2.131^{a}	27.942^{c}	4.259^{bc}	18.770^{c}	5.204^{a}	32.041^{c}	18.992^{b}	19.057^{b}
SEM	0.006	0.096	0.157	8.443	2.358	0.034	14.718	0.805

[a, b, c, d, e] Vaules in the same column without the same superscripts are significantly different ($p<0.05$, n = 8).

contrast, no substrate mixture had a good protection effect on protease activity in simulations of total tract digestion.

Amylase and protease : Starch and soybean protein mixed substrate (SS) provided the best protection for amylase in simulations of monogastric animal and avian stomach and avian total tract digestion (Table 3); the residual amylase activity was about 10 times higher than that of the enzyme mixture without substrate addition (EN). No any substrate mixture could provide effective protection to protease in simulations of total tract digestion for both animal modules (Table 3).

Non-purified substrate protection ability tests

BSA : In simulations of stomach digestion, individual cellulase, protease or amylase were stabilized by addition of >1.5% BSA (Table 4). However, BSA addition had no protection on protease activity in simulation of total tract digestion for both animal modules. Protection was greater for cellulase than other enzymes when BSA was added with mixed enzymes, especially in simulations of total tract digestion. Addition of 1.5% BSA seemed capable of protecting mixed enzymes in simulation of stomach digestion Compared with other protease substrates in this study (casein, gelatin or soybean protein), BSA had a greater protective effect on cellulase than other individual protein substrates in mixed enzymes containing protease (Table 2; EC, EG and ES treatment).

BSA is a soluble protein known to be resistant to

Table 4. The protective effects of BSA

BSA %	Cellulase (glucose μg/mg/min)		Protease (azocasein mg/mg/min)		Amylase (p-nitrophenol μg/mg/h)	
	Individual enzyme	Mixed enzymes	Individual enzyme	Mixed enzymes	Individual enzyme	Mixed enzymes
Avian stomach simulation						
0.25	56.583^{e}	121.204^{bc}	1.074^{d}	1.728^{ab}	13.006^{ab}	32.679^{a}
0.5	65.916^{d}	115.860^{c}	1.611^{c}	2.316^{a}	15.546^{ab}	32.387^{a}
1.0	81.958^{c}	127.011^{ab}	2.179^{b}	1.604^{b}	12.498^{b}	33.116^{a}
1.5	108.500^{b}	132.355^{a}	2.567^{a}	1.552^{b}	18.595^{a}	29.474^{b}
2.0	119.583^{a}	134.911^{a}	2.478^{a}	1.687^{b}	14.022^{ab}	29.037^{b}
Blank	45.500^{f}	127.012^{ab}	0.578^{e}	1.368^{b}	19.103^{a}	26.706^{c}
SEM	3.763	21.501	0.006	0.074	4.004	0.797
Avian total tract simulation						
0.25	269.618^{cd}	330.331^{ab}	33.176	24.241	12.177^{c}	23.007
0.5	264.236^{d}	338.767^{ab}	33.045	20.897	14.495^{a}	21.446
1.0	289.062^{bc}	333.789^{ab}	33.869	24.976	12.581^{bc}	22.053
1.5	307.812^{b}	344.990^{a}	32.927	25.439	14.899^{a}	28.297
2.0	332.291^{a}	326.045^{ab}	32.404	25.253	13.891^{ab}	21.099
Blank	262.326^{d}	324.108^{b}	32.454	24.368	14.093^{a}	19.539
SEM	71.166	67.152	0.505	8.684	0.303	13.562
Monogastric animal stomach simulation						
0.25	62.65^{d}	122.966^{e}	1.143^{cd}	1.201^{a}	13.033^{a}	14.213^{b}
0.5	69.941^{d}	151.841^{d}	1.233^{bcd}	1.321^{a}	11.866^{ab}	13.908^{b}
1.0	91.525^{c}	171.675^{c}	2.279^{a}	1.309^{a}	12.533^{ab}	13.909^{b}
1.5	107.858^{b}	192.383^{b}	1.759^{ab}	1.275^{a}	12.033^{ab}	15.735^{a}
2.0	122.733^{a}	214.55^{a}	1.502^{bc}	1.361^{a}	11.533^{ab}	14.822^{ab}
Blank	48.358^{e}	120.05^{e}	0.886^{d}	0.885^{b}	11.033^{b}	12.387^{c}
SEM	24.061	30.651	0.047	0.009	0.439	0.236
Monogastric animal total tract simulation						
0.25	245.972^{c}	309.4^{ab}	31.768	25.826	35.138^{c}	37.53^{b}
0.5	240.243^{c}	314.094^{ab}	31.227	24.845	37.916^{a}	37.059^{b}
1.0	259.687^{bc}	324.566^{ab}	31.583	25.947	42.976^{bc}	39.226^{b}
1.5	293.02^{ab}	337.025^{ab}	31.754	25.664	36.13^{a}	42.052^{ab}
2.0	303.958^{a}	346.233^{a}	32.067	26.018	28.789^{ab}	55.617^{a}
Blank	239.895^{c}	294.955^{b}	30.401	26.036	34.742^{a}	34.798^{b}
SEM	218.737	328.434	0.708	0.278	25.436	37.891

$^{a, b, c, d, e, f}$ Means in the same column without the same superscripts are significantly different ($p<0.05$, n = 8).

proteolysis, and has a complex tertiary structure with 6% cysteine and disulphide bonds (Broderick and Craig, 1989). Casein has essentially a linear secondary and tertiary structure without disulphide bonds, and was therefore sensitive to degradation (Mangan, 1972). This may explain why BSA had better protection of enzyme residual activity than casein. As BSA was added to the mixture of cellulase, protease and amylase, it is possible that the BSA could bind to protease and prevent protease from attacking cellulase or amylase.

Soybean hull : The simulation of stomach digestion indicated that soybean hull addition was useful to protect the residual activity of all three kinds of enzyme in the avian module (Table 5), but it had no significant protection effect on amylase in the monogastric animal module. Addition of 1.5% soybean hull appeared to significantly protect cellulase activity applied alone or in mixed enzymes in simulations of total tract digestion for both animal modules (Table 5). Compared to BSA, soybean hull seems to have greater potential for protecting exogenous cellulase in digestion simulation tests (Tables 4 and 5). There was no noticeable protection of protease and amylase residual activity when soybean hull was added to individual or mixed enzymes.

There are a number of components present in soybeans that can exert a negative impact on animal nutrition. These include protease inhibitors, lectins, saponins, tannins, phytate and other factors (Liener, 1994). Saponin content of soybean hull was reported to be about 2% on a dry matter basis (Price et al., 1987). Soybean saponins can inhibit pancreatic enzymes by a nonspecific interaction (Ishaaya and Birk, 1965). Ikedo et al. (1996) indicated that BSA susceptibility to chymotrypsin digestion was decreased by soybean saponins and that the N-terminal peptide fragment

Table 5. The protective effects of soybean hull

Hull %	Cellulase (glucose μg/mg/min)		Protease (azocasein mg/mg/min)		Amylase (p-nitrophenol μg/mg/h)	
	Individual enzyme	Mixed enzymes	Individual enzyme	Mixed enzymes	Individual enzyme	Mixed enzymes
Avian stomach simulation						
0.25	62.417^{e}	117.075^{d}	1.923^{d}	1.797^{c}	22.884^{c}	20.300^{d}
0.5	81.375^{d}	125.825^{cd}	1.975^{d}	1.881^{bc}	23.193^{c}	26.027^{cd}
1.0	107.625^{c}	133.992^{cd}	2.125^{cd}	1.977^{bc}	24.428^{c}	31.595^{bc}
1.5	144.958^{b}	162.283^{b}	2.773^{a}	2.157^{a}	27.053^{b}	37.164^{ab}
2.0	170.333^{a}	187.367^{a}	2.524^{ab}	2.129^{a}	29.369^{a}	40.505^{a}
Blank	51.333^{e}	96.367^{e}	2.322^{bc}	1.819^{c}	19.796^{d}	23.959^{d}
SEM	36.260	22.846	0.011	0.002	0.642	6.946
Avian total tract simulation						
0.25	279.340^{c}	325.420^{b}	30.608^{a}	31.943^{b}	21.158^{c}	18.712^{d}
0.5	287.330^{bc}	339.860^{b}	29.159^{b}	32.526^{ab}	21.158^{c}	21.837^{cd}
1.0	315.970^{b}	353.190^{b}	28.726^{b}	33.018^{ab}	23.548^{b}	24.489^{bc}
1.5	373.780^{a}	391.040^{a}	28.464^{b}	32.385^{ab}	24.835^{ab}	29.129^{ab}
2.0	376.040^{a}	420.030^{a}	28.209^{b}	32.124^{ab}	26.857^{ab}	31.402^{a}
Blank	291.490^{bc}	328.190^{b}	28.268^{b}	32.338^{ab}	18.493^{d}	19.375^{cd}
SEM	143.771	183.528	0.214	0.125	0.735	3.994
Monogastric animal stomach simulation						
0.25	59.303^{e}	102.520^{bc}	0.430^{b}	0.451^{b}	12.141	8.559
0.5	68.819^{d}	107.040^{bc}	0.565^{b}	0.571^{b}	15.702	9.154
1.0	87.741^{c}	140.580^{ab}	0.669^{b}	0.615^{ab}	20.248	10.123
1.5	99.991^{b}	160.120^{a}	0.660^{b}	0.787^{a}	22.512	11.542
2.0	121.538^{a}	179.520^{a}	1.047^{a}	0.799^{a}	24.379	11.766
Blank	51.756^{e}	88.960^{c}	0.394^{b}	0.521^{b}	20.936	8.921
SEM	22.735	77.726	0.025	0.011	13.082	6.176
Monogastric animal total tract simulation						
0.25	209.710^{bc}	328.300^{b}	29.816^{b}	29.422^{ab}	33.258	41.566
0.5	218.570^{b}	328.300^{b}	30.449^{ab}	29.475^{ab}	28.384	39.743
1.0	228.200^{b}	386.280^{ab}	30.810^{a}	29.833^{a}	32.356	40.769
1.5	267.460^{a}	442.270^{a}	30.721^{ab}	31.088^{a}	31.181	31.198
2.0	286.540^{a}	435.070^{a}	30.467^{ab}	30.824^{a}	33.460	40.325
Blank	194.870^{c}	315.190^{b}	28.786^{c}	27.563^{b}	31.545	46.970
SEM	163.875	284.831	0.277	2.763	4.541	5.008

$^{a, b, c, d, e}$ Means in the same column without the same superscripts are significantly different ($p<0.05$, $n = 8$)

obtained from the hydrolysate of BSA-soya saponin complex could interact with soya saponin to form a protease-resistant moiety that had low sensitivity to chymotrypsin. It is possible that the protective effect of soybean hull observed in the present study may be related to the inhibition of digestive enzyme by saponins. High levels of pectin in soybean hull might be another protection factor because pectin can increase the viscosity of solution and hinder the process of digestion (Atallah and Melnik, 1982).

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