Stability of feed enzymes in physiological conditions assayed by *in vitro* methods

JOHAN INBORR and ANNE GRÖNLUND

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A series of *in vitro* incubations were carried out to investigate the stability of two enzyme preparations in conditions similar to those in the upper gastrointestinal tract of monogastric animals. The two enzyme products, one crude xylanase from *Trichoderma longibrachiatum* (Multifekt K) and the other a specifically manufactured feed enzyme (Avizyme SX®), were subjected to incubations at low and neutral pH with and without proteolytic enzymes (pepsin and pancreatin). Wheat gluten was employed together with the crude xylanase to investigate its potential as a stabilising agent. Due to the buffering effect of Avizyme SX®, incubations were carried out with (pH 2.5) and without (pH 3.2) addition of either citric or hydrochloric acid. Incubation of the crude xylanase at low pH followed by incubation at neutral pH resulted in negligible loss of xylanase activity whereas β-xylosidase recovery fell to 57 per cent of the initial value ($P<0.05$). Addition of wheat gluten resulted in full recovery of β-xylosidase. The recoveries of both β-glucanase and xylanase were significantly ($P<0.05$) lower than the initial values after incubation of Avizyme SX® in pH 2.5. However, with no pH adjustment (pH 3.2) the recoveries were significantly higher ($P<0.05$ for β-glucanase and $P<0.10$ for xylanase). The results from the pepsin and pancreatin incubations showed similar trends as the ones of the pH stability experiments. Consequently, gluten addition and no pH adjustment gave the highest enzyme activity recoveries.

The results suggest that partial enzyme inactivation may occur due to the low pH and proteolytic activities and hence in the GI-tract of monogastrics. Feeds and feedstuffs can due to their buffering capacity and possibly by providing substrates for the enzymes markedly reduce the rate of inactivation. Results from a number of pig and poultry experiments appear to support this assumption. *In vivo* recovery measurements using animal models are needed to substantiate this.

Key words: xylanase, β-glucanase, β-xylosidase, wheat gluten, enzyme survivability, pH, pepsin, pancreatin, Multifect K, Avizyme SX®.

Introduction

Since the early 1980's the use of enzymes in animal feeds has received much attention both in the academic and commercial worlds. There is now a considerable amount of published work showing the response in animal performance to enzyme supplementation of feeds, which has been extensively reviewed by CHESSON (1987) and DIERICK (1990). With increased commercial use of feed enzymes stability has become a frequently debated issue. In particular, enzyme stability during feed manufac-

turing, due to the different heat treatments (pelleting, expansion, extrusion etc.) now being employed in the feed industry, has caused some concern. Furthermore, once the enzymes enter into the gastrointestinal tract, they are subjected to a range of conditions that can cause inactivation. Firstly, gastric pH may be as low as 1, but usually around 2 in an empty stomach, with rapid increase to between 4 and 5 as ingested food arrives (KIDDER and MANNERS 1978). Keeping in mind that enzymes are proteins and, therefore, subject to proteolysis, low pH in combination with pepsin would appear to be a potential hazard for the enzyme molecules. Secondly, the pancreatic proteases e.g. trypsin and chymotrypsin represent a major threat to the enzymes due to their abundance and high activity in duodenum.

Cellulases and hemicellulases are the most commonly used enzymes in feed applications. These are mainly microbial enzymes produced by different fungi and bacteria. In general, the inherent heat stability of mesophilic microbial enzymes is poor, although there are considerable differences between enzymes from different sources in this respect (GODFREY and REICHELT 1983). Through modern biotechnology a number of stabilisation methods have been developed. For example, it is a known fact that substrates and inhibitors that bind specifically to the native conformation of an enzyme will increase the stability (PACE 1990). This method of stabilisation is called immobilisation and has been shown by SIMONS and GEORGATSOS (1990) to decrease heat inactivation of barley β-glucosidase. Other promising stabilisation methods involve adding crosslinks (disulfide bonds) to, substituting amino acids (PACE 1990) and crystallisation of the enzyme proteins (ST. CLAIR and NAVIA 1992). Furthermore, DE CORDT et al. (1992) found that polyols and carbohydrates are powerful stabilisers for both dissolved and immobilised *B. licheniformis* α-amylase.

A series of *in vitro* experiments were designed to investigate the survivability of a few main activities of two enzymes subjected to conditions similar to those prevalent in the digestive tract of pigs and poultry. Two enzyme preparations, one crude and one specifically manufactured feed enzyme product, were employed. Xylanase and ß-xylosidase activities of the crude enzyme preparation and ß-glucanase and xylanase of the feed enzyme product were investigated.

Material and methods

A liquid crude xylanase preparation (Multifect K, Genencor International Ltd., Helsinki, Finland) from a *Trichoderma longibrachiatum* fermentation and a stabilised (liquid enzymes adsorbed and dried onto a wheat-based carrier; Patent FI 77359) commercial feed enzyme product (Avizyme SX®, Finnfeeds International Ltd., Marlborough, Wiltshire, United Kingdom) containing β-glucanase and xylanase from *Trichoderma longibrachiatum* were subjected to treatments in conditions similar to those in the stomach (proventriculus) and small intestine of pigs and poultry. A schematic descriptions of the incubation procedure employed is presented in Figure 1.

Incubation in low and neutral pH

The conditions employed in these incubations were identical with those described below with the exception that no pepsin and pancreatin were used.

Incubation with pepsin and pancreatin

Crude xylanase preparation

20 ml of pepsin solution (Merck 7189), containing 0.25 protease units (haemoglobin, pH 1.2, 25°C) per ml, in 0.1 M McIlvaine's buffer (pH 2.5) was equilibrated at 40°C. 10 ml of xylanase solution in McIlvaine's buffer (1 ml xylanase/50 ml buffer) was added and the solution diluted with buffer to 50 ml followed by incubation at 40°C, pH 2.5 for 30 minutes. The reaction was stopped in an ice/water bath immediately after incubation. pH was adjusted

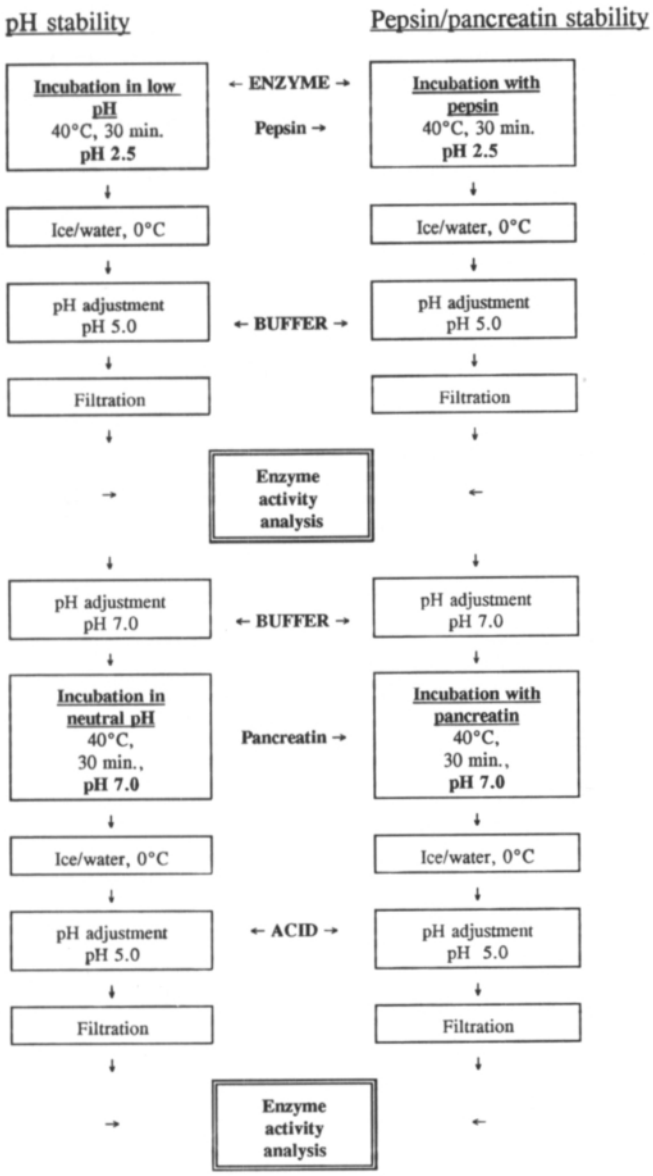

Fig. 1. Flow chart diagramme of the incubations.

with 0.5 M Na_2HPO_4 to 5.0 and the final volume was 100 ml. Xylanase and β-xylosidase activities were measured. The pepsin treated sample (10 ml) was then adjusted to pH 7.0 with 0.5 M Na_2HPO_4. 30 ml of pancreatin solution (Merck 7230), containing 30 protease units per ml, in McIlvaine's buffer (pH 7.0), was added, and the solution diluted to 50 ml and adjusted to pH 7.0 with McIlvaine's buffer. The solution was incubated at 40°C for 30 minutes, after which time the reaction was stopped in an ice/water bath. The pH was adjusted to 5.0 by 0.5 M citric acid and xylanase and β-xylosidase activities measured.

A parallel incubation was carried out with wheat gluten (2% w/v) solubilised in a NaCl solution (0.9% w/v) as a stabiliser. When wheat gluten was

used, samples were filtered through fibre glass filter paper before enzyme activity analysis.

Commercial feed enzyme

Two proteolysis procedures were carried out. In the first one, the pH was kept constant at 2.5 by addition of 6 N HCl, whereas in the second one no acid was added during the pepsin treatment, and the pH rose to 3.2 (buffering effect of the feed enzyme product).

20 g feed enzyme premix was suspended in 0.1 M McIlvaine's buffer (pH 2.5) and the pH was either maintained at 2.5 by acid (6 N HCl) addition or allowed to rise due to the buffering capacity of the feed enzyme product (parallel incubation). 10 ml pepsin solution was added to give a final volume of 70 ml. The pH was adjusted after incubation to 5.0 using 2.5 M NaOH and samples were taken for enzyme activity analysis (β-glucanase and xylanase) after filtration through fibre glass filter paper.

The procedure continued with 70 ml unfiltered pepsin-treated sample, employing the same conditions as with the crude xylanase preparation but without addition of wheat gluten. 15 ml pancreatin solution in 0.1 ml McIlvaine's buffer (pH 7.0) was added giving a volume of 85 ml during the incubation. After the pH adjustment (15 ml 2 M citric acid) the final volume was 100 ml. β-glucanase and xylanase activities were measured after filtration.

Enzyme activity analysis

β-glucanase (EC 3.2.1.6; endo-β-(1,3)(1,4)-glucanase) activity was determined spectrophotometrically using 1.0% barley β-glucan (Biocon Biochemicals Ltd., Ireland) in McIlvaine's buffer at pH 5.0 as substrate. 0.2 ml of suitable enzyme dilution in deionized water was incubated with 2.0 ml of substrate solution at 40°C for 30 minutes. Reducing sugars were assayed by addition of 3.0 ml 3,5-dinitrosalisylic acid (DNS) reagent (SUMNER and SOMERS 1949), boiling for 5 minutes, cooling and measuring absorbance at 540 nm. One β-glucanase unit is the amount of enzyme that liberates 1 μmol of reducing sugars (expressed as glucose equivalents) in one minute under the conditions described.

Xylanase (EC 3.2.1.8; endo-β-(1,4)-xylanase) activity (modified from POUTANEN et al. 1986) was determined using 1% oat spelt xylan (Sigma X-0376) in McIlvaine's buffer at pH 5.0 as substrate. 0.2 ml of suitable enzyme dilution was incubated with 2.0 ml of substrate solution at 40°C for 30 minutes. The reducing sugars were assayed as described above. One xylanase unit is the amount of enzyme that liberates 1 μmol of reducing sugars (expressed as xylose equivalents) in one minute under the conditions described.

β-xylosidase (EC 3.2.1.37; 1,4-β-D-xylan xylohydrolase) activity was determined (modified from DELEYN et al. 1978) using 2 mmol/l p-nitrophenyl-β-D-xylopyranoside in McIlvaine's buffer at pH 5.0 as substrate. 0.2 ml of suitable enzyme dilution in deionized water was incubated with 2.0 ml substrate solution in 40°C at pH 5.0 for 30 minutes. After adding 1.0 ml of Na_2CO_3, absorbance was measured at 400 nm. One β-xylosidase unit is the amount of enzyme that liberates one μmol of p-nitrophenol in one minute under the conditions described.

Each enzyme activity measurement was carried out in duplicates or triplicates unless otherwise stated. Values are expressed as means with standard deviations of each sample. Means were separated where appropriate by paired t-test.

Results and discussion

pH stability

Incubation in pH 2.5 did not reduce the xylanase activity of Multifect K, whereas the β-xylosidase activity was reduced to 57% ($P<0.05$) of the initial value (Table 1). Subsequent incubation at pH 7 only marginally reduced xylanase activity and had no effect on β-xylosidase. Addition of wheat gluten markedly increased the recovery of β-xylosidase after incubation in pH 2.5 ($P<0.05$) and pH 7 ($P<0.10$), resulting in full recovery of this activity.

Maintaining pH at 2.5 by addition of citric acid

Table 1. Initial and residual xylanase and β-xylosidase activities of Multifect K, measured at pH 5, after incubation at acidic and neutral pH with and without pepsin and pancreatin, and with (+) and without (-) gluten (means ± sd).

Gluten		Xylanase				β-xylosidase			
		-		+		-		+	
		U/g	%	U/g	%	U/g	%	U/g	%
Initial	pH 5	4510 ± 80	100	4970 ± 27	100	44 ± 3	100	30 ± 2	100
Residual	pH 2.5	4440*	98	4780*	96	25^{y} ± 1	57	30^{a} ± 1	100
	pH 7	4070 ± 235	90	4720*	95	25^{y} ± 1	57	30^{b} ± 2	100
Pepsin	pH 2.5	4270*	95	4520*	91	30^{y} ± 2	68	18^{y} ± 0	60
Pancreatin	pH 7	2590^{x} ± 4	57	3400*	68	25 ± 5	57	29 ± 2	97

* result of one measurement
[x] differ from initial value ($P<0.05$)
[y] differ from initial value ($P<0.10$)
[a] differ from corresponding control (+) ($P<0.05$)
[b] differ from corresponding control (+) ($P<0.10$)

Table 2. Initial and residual β-glucanase and xylanase activities of Avizyme SX, measured at pH 5, after incubation at acidic and neutral pH with and without pepsin and pancreatin, and with (+) and without (-) pH adjustment (means ± sd).

pH adjustment[1]		β-glucanase				Xylanase			
		+		-		+		-	
		U/g	%	U/g	%	U/g	%	U/g	%
Initial	pH 5	870 ± 5	100	850 ± 15	100	460 ± 16	100	467 ± 38	100
Residual	pH 2.5/3.2	370^{x} ± 6	43	830^{a} ± 28	96	340^{y} ± 6	75	510^{b} ± 20	109
	pH 7	390^{x} ± 8	45	790^{a} ± 28	92	260^{xz} ± 2	57	422^{a} ± 6	90
Pepsin	pH 2.5/3.2	370^{x} ± 17	42	850^{a} ± 13	100	360^{y} ± 3	78	510^{b} ± 18	110
Pancreatin	pH 7	310^{x} ± 9	36	760^{a} ± 30	89	260^{xz} ± 12	56	470^{b} ± 21	100

[1] (+) means pH kept constant at 2.5 by addition of either 2 M citric acid (pH stability) or 6 N HCl (pepsin stability), (-) means no acid addition during the incubation with pH rising to 3.2.
[x] differ from initial value ($P<0.05$)
[y] differ from initial value ($P<0.10$)
[z] differ from pH 2.5 ($P<0.05$)
[a] differ from corresponding control (+) ($P<0.05$)
[b] differ from corresponding control (+) ($P<0.10$)

resulted in 43% ($P<0.05$) recovery of β-glucanase and 75% recovery of xylanase ($P<0.10$) in Avizyme SX® (Table 2). Subsequent incubation at pH 7 had no effect on the β-glucanase activity, whereas xylanase was reduced to 57% ($P<0.05$) of the initial activity. When pH was not adjusted, leading to a 0.7 unit increase in pH, β-glucanase recovery after the first incubation (pepsin in pH 3.2) was 96% ($P>0.05$) and after the second incubation (pancreatin in pH 7) 92%. The corresponding recoveries for xylanase were 109 and 90%. This difference was not significant ($P>0.10$).

From these results it appears that low pH *per se* did not cause any dramatic reductions in enzyme activity and that addition of wheat gluten may reduce the degree of inactivation of more sensitive enzymes i.e. in this case β-xylosidase. Whether this effect is due to an increased dry matter content of

the system or e.g. binding between the gluten and the enzyme is not known. Interestingly, with wheat being the carrier material of Avizyme SX® thus providing the system with gluten, xylanase recoveries were lower with and higher without pH adjustment than when gluten was added to Multifect K.

Pepsin and pancreatin stability

Xylanase and β-xylosidase recoveries after incubation of Multifect K with pepsin was 95 and 68% without and 91 and 60% with added gluten, respectively (Table 1). These effects were not significant ($P>0.10$), with the exception being β-xylosidase when incubated at pH 2.5 with added gluten ($P<0.10$). After the subsequent incubation in pancreatin the corresponding recoveries were 57 and 57% and 68 and 97%, respectively ($P>0.10$). Thus β-xylosidase seemed to be more sensitive to pepsin than xylanase. Incubation with pancreatin tended to further reduce enzyme activity, except the activity of β-xylosidase when gluten was added.

β-glucanase activity of Avizyme SX® decreased to 42% ($P<0.05$) of the initial value after incubation in pepsin with pH kept at 2.5 by addition of hydrochloric acid and was further reduced to 36% after incubation in pancreatin (Table 2). With no pH adjustment, the β-glucanase recoveries after pepsin treatment was 100% and after incubation with pancreatin 89% ($P>0.10$). These recoveries were significantly higher than when pH was kept at 2.5 ($P<0.05$). Xylanase recoveries after pepsin and pancreatin incubations with pH adjustment were 78 and 56% of the initial activity ($P<0.10$), respectively. Without pH adjustment xylanase was fully recovered. These recoveries were significantly higher than with pH adjustment ($P<0.10$).

Addition of wheat gluten to the enzyme/substrate systems tended to increase the final enzyme recoveries but this effect was not observed at pH 2.5, except for β-xylosidase after incubation at pH 2.5 without pepsin. DE CORDT et al. (1992) found that both polyols and carbohydrates increased the temperature stability of bacterial α-amylase. The mechanisms involved were not specifically elucidated but "preferential protein hydration", changes in the chemical potential of the proteins, changes in the solvent dielectric constant, changes in the water activity of the solvent system, degree of water organisation were suggested. It is possible that wheat gluten used in this present experiment exerted one of these effects leading to increased pH and proteolytic stability. Interestingly, the buffering capacity of the feed enzyme product, probably due to the cereal carrier material, had a clear stabilising effect on the enzyme activities measured. Feed arriving in the stomach of pigs exert similar pH buffering effects (KIDDER and MANNERS 1978) and it can therefore be assumed that the feed acts as a potent stabilizer in the animal.

Results from a number of experiments with pigs and poultry would suggest that sufficient activity of supplementary enzymes survive the potential hazards of the GI-tract to improve animal performance (CHESSON 1987, DIERICK 1990). Enzyme recovery measurements in different segments of the GI-tract would be needed to exactly establish the rate and extent of feed enzyme inactivation *in vivo*.

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Johan Inborr
Finnfeeds International Ltd.
Market House, High Street
Marlborough, Wiltshire SN8 1AA
United Kingdom

Present address:
Finnsugar Gmbh
Raboisen 58
D-20095 Hamburg 1, Germany

Anne Grönlund
Cultor Ltd.,Technology Centre
FIN-02640 Kantvik, Finland

SELOSTUS

Rehuentsyymien stabiilisuus fysiologisissa olosuhteissa *in vitro* -menetelmin mitattuna

JOHAN INBORR ja ANNE GRÖNLUND

Finnfeeds International Ltd. ja Cultor Ltd.

Kahden entsyymipreparaatin, *Trichoderma longibrachiatum*in tuottaman ksylanaasin (Multifekt K) ja rehuentsyymituotteen (Avizyme SX®), stabiilisuutta tutkittiin *in vitro* -menetelmin sellaisissa olosuhteissa, jotka vallitsevat yksimahaisten ruoansulatuskanavan yläosissa. Ksylanaasipreparaatista tarkasteltiin ksylanaasi- ja β-ksylosidaasiaktiivisuuksien-, sekä Avizyme SX®:stä β-glukanaasi- ja ksylanaasiaktiivisuuksien stabiilisuutta. Aluksi tutkittiin entsyymiaktiivisuuksien stabiilisuutta eri pH:ssa (2,5 ja 7) ja sen jälkeen pepsiinin ja pankreatiinin vaikutusta entsyymiaktiivisuuksiin. Tutkimuksessa selvitettiin myös vehnägluteenin entsyymiaktiivisuuksia stabiloivaa vaikutusta.

Inkubointi pH 2,5:ssä ja sen jälkeen pH 7:ssä ei vaikuttanut merkitsevästi Multifekt K:n ksylanaasiaktiivisuuteen. Sen sijaan β-ksylosidaasiaktiivisuus aleni 57 %:iin alkutasosta ($P<0.05$) ilman vehnägluteenilisäystä. Vehnägluteenilisäyksellä β-ksylosidaasiaktiivisuus pysyi alkutasolla. Avizyme SX®:n β-glukanaasi- ja ksylanaasiaktiivisuus aleni merkitsevästi kun sitruunahappoa lisättiin inkuboinneissa. Ilman happolisäystä enstyymiaktiivsuudet pysyivät alkutasoilla.

Inkubointi pepsiinillä ja pankreatiinilla alensi merkitsevästi ($P<0.05$) Multifekt K:n ksylanaasiaktiivisuutta. β-ksylosidaasi aleni 68 %:iin pepsiini-inkuboinnin jälkeen ($P<0.10$) ja 57 %:iin pankreatiini-inkuboinnin jälkeen ($P>0.10$). Pepsiiniinkuboinnissa vehnägluteenilisäys ei parantanut stabiilisuutta, mutta pankreatiini-inkuboinnissa vehnägluteenin lisäys nosti β-ksylosidaasiaktiivisuuden 97 %:iin alkutasosta. Avizyme SX®:n pepsiini- ja pankreatiini-inkuboinnit ja happolisäys alensivat merkitsevästi ($P<0.05$) β-glukanaasi- ja ksylanaasiaktiivisuutta. Ilman happolisäystä aktiivisuudet pysyivät alkuperäisillä tasoilla.

Tulokset osoittavat, että entsyymien osittainen inaktivoituminen voi tapahtua matalassa pH:ssa ja proteolyyttisissä olosuhteissa. Avizyme SX®:llä ja vehnägluteenilisäyksellä saatujen tulosten perusteella on kuitenkin odotettavaa, että rehun mahalaukun sisältöä puskuroiva vaikutus suojaa rehuun lisättyjä entsyymejä inaktivoitumiselta suhteellisen tehokkaasti. Rehuun lisättyjen entsyymien todellisen inaktivoitumisasteen määrittämiseksi joudutaan kuitenkin suorittamaan vastaavat mittaukset kohde-eläimiä käyttämällä.