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DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD  
Secondary Reviewer: Michael Watson, Ph.D., Plant Pathologist, BPPD

STUDY TYPE: *In vitro* Digestibility and Bioavailability in the Rodent  
MRID NO: 447343-05  
CHEMICAL NO: 006466  
TEST MATERIAL: Cry9C protein from *Bacillus thuringiensis* ssp. *tolworthi*  
STUDY NO: RIKILT-DLO No. 71.113.04.D97.2-3  
SPONSOR: AgrEvo USA Company, Wilmington, DE  
TESTING FACILITY: RIKILT-DLO, Netherlands  
TITLE OF REPORT: Assessment of the Stability to Digestion and Bioavailability of the Lys Mutant Cry9C Protein from *Bacillus thuringiensis* serovar *Tolworthi*

AUTHOR: Sally Van Wert, Ph.D.; Dr. H.P.J.M. Noteborn

STUDY COMPLETED: 16 September 1998

CONCLUSION: All three batches of Lys mutant Cry9C were shown by SDS-PAGE and western blot to be substantially made up of Cry9C protein compared to the 100% standard provided by PGS. Greater than 90% of each preparation had the 68 kDa band as the major component with the majority of the remaining contaminant being the 55 kDa degradation form. The three batches of Lys mutant Cry9C either from *E.coli* (batches I & II) or *B. thuringiensis* (batch III) were shown to be substantially resistant to degradation after 2 hours by either SGF or SIF. When the Cry9C samples were subjected to heat (either room temperature 20°C or 90°C) for extended periods, the protein appeared to be stable as the protein banding pattern in SDS-PAGE gels was unaltered. The presence of a tomato matrix does not appear to affect this heat stability. However, the company has presented an isoelectric focusing gel which purports to show a loss of detectable protein forms after 30 to 60 minutes. The data for bioavailability of Cry9C protein was monitored by the indirect double antibody sandwich ELISA previously described which had a detection limit of 2.0 ngCry9C/ml in serum with a recovery rate of 85±5%. Previous work with Cry1Ab5 is also presented and that ELISA had a detection limit of 5.0 ngCry1Ab5/ml with a recovery rate of 55±5%. The animals were dosed with a range of Cry9C from 2.6 to 298 mg/kg bodyweight by gastric intubation. All the dosed animals appeared and behaved normally, survived to sacrifice and displayed no treatment related adverse effects. No ELISA positive Cry9C protein or fragments were detected in blood samples taken from rats at the 2.6mg/kg dose. At the higher dose rates, Cry9C ELISA values were positive giving a calculated value of between 5 and 15 ng Cry9C/ml in serum. The absorption rate was not affected by dose amount or presence of a food matrix. The percentage of bioavailable Cry9C

was calculated to range from 0.0006% (42mg/kg) to 0.0002% (298mg/kg) of the dose administered. More importantly, an SDS-PAGE/western blot confirmation of the sera that were ELISA positive for the presence of Cry9C protein showed that only one of the rats had serum with confirmed Cry9C protein. This rat (#548) had a faint Cry9C positive band, all the other sera had no detectable Cry9C reactivities. No interfering compounds were found in rat serum or BSA. The positive signals were attributed to cross-reaction with Cry9C like components present in both control and Bt-treated animals since there were no significant differences between the 2-D electrophoresis or ECL results. This data indicating cross reaction was not presented.

**CLASSIFICATION:**

Acceptable.

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**STUDY DESIGN**

Examination of the stability of the Lys mutant Cry9C to *in vitro* digestive solutions and *in vivo* bioavailability and mucosal binding of the Lys mutant Cry9C in rats. The study was not conducted according to the GLP standards in 40 CFR 160 or the OECD Council Decision of 12 May 1981. The report is also being claimed as confidential by RIKILT-DLO but not by AgrEvo. AgrEvo's statement on data confidentiality also states that AgrEvo's claim supercedes all other confidentiality statements in the report.

Test materials: Three batches of the Cry9C toxin test substance (EMBL Z37527) was provided by Plant Genetic Systems (Ghent, Belgium). The batches were stated as being 100% pure by western blot analysis. The amounts were stated as 3.0mg/ml buffer; 2.6mg/ml buffer and 1.1 g of freeze-dried powder, respectively. Reference toxins CryI Ab5 (0.7mg/ml) and CryIIIb (2.1mg/ml) were provided by PGS and Metapontum Agrobios (Italy), respectively.

Certain chemical reagents were also required for the performance of these tests. All were reagent grade obtained from commercial sources except for the following special notations: bovine serum albumin fraction V, trypsin inhibitor, porcine pancreatin, goat anti-rabbit IgG-antibody conjugate to alkaline phosphatase, 5,5-dithiobis-2-nitrobenzoic acid (DNTB), acetylCoA from Sigma Chemical, St. Louis, USA. Trypsin and pepsin from porcine gastric mucosa were obtained from Boehringer Mannheim, Ingelheim, Germany. Tetramethylbenzidine and 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) were from KPL, Inc. Gaithersburg, USA. <sup>3</sup>H-propranolol and <sup>14</sup>C-PEG were from Amersham, UK. Lugol was from Fluka, Switzerland, Diaminobenzidine from Pierce, USA and the deionized water from a MilliQ unit Millipore, Bedford, USA.

Test animals: Fifty-two Wu/Wister rats were obtained from a specific pathogen free colony at the Harlan facility in Germany. While being kept in the Central Experimental Animal Accommodation (Agricultural University, Wageninagen, the Netherlands), the rats were checked for overt signs of ill health or anomalies and assigned a unique cage with a card including the animal identification number, cage number, group letter and study number. During the study each animal was assigned a cage maintained in the animal facility at 21±1 °C with a controlled humidity of 55±10%, artificial

light for 12 hours each day (7:70AM to 7:30PM) and supplied with 10 changes of air per hour. The rats had an average bodyweight of 280g at the start of the experiment and were provided food (basal rodent diet, RMH-B; Hope Farms Woerden, the Netherlands) and water *ad libitum* unless some laboratory functions precluded its advisability.

**Test methods: Protein concentration** was determined with a BCA protein assay kit from Pierce using BSA as a standard. Samples were taken from remaining stock solutions that were kept at -40 to -80°C. The samples were also subjected to western blotting to check stability, purity and composition.

**In vitro digestibility** was done in both simulated gastric (SGF) and intestinal fluids (SIF) prepared as described in the U.S. Pharmacopeia (vol. XXII, p.1788-1789). The SGF was 32mg of pepsin diluted in 10ml of gastric buffer (0.5g NaCl, 1.75ml of 1.0M HCl in 250ml water, pH 2.0) and the SIF was 0.2g pancreatin diluted in 20ml of intestinal fluid (0.17g KH<sub>2</sub>PO<sub>4</sub>, 4.7ml of 0.2M NaOH in 20ml of water). The proteins were first subjected to pepsin digestion for up to 2 hours at 37°C then to pancreatin activity for up to 4 hours at 37°C. The proteins were added to 1.0ml of SGF or SIF at a concentration of 50-165µg/ml, respectively. At given time points 50µl aliquots were removed, quenched (Fuchs *et al.*, 1993, Bio/Technology 11:1543-1547) and stored at -40°C until the digestive fate of the proteins of interest and the reference standards were assayed. SGF was monitored for activity by an assay which measured the increase in absorbance at 280nm following TCA precipitation of the SGF digested hemoglobin.

**Thermostability testing** involved diluting protein to a 0.7 to 0.8mg/ml concentration in water, buffer or tomato matrices. Fifty µl samples were exposed to processing temperatures of from 50 up to 90°C for from 5 to 210 minutes. After the heat treatment the samples were immediately put on ice then frozen to -40°C until analyzed further.

**Bioavailability testing** required the use of rats with indwelling cannula of the vena porta inserted as described in the literature (Remie *et al.*, 1990, Manual of Microsurgery on the Laboratory Rat, Vol. IV, p.213-230, Elsevier) while employing as anesthesia 0.5ml hypnorm (s.c.), 0.5ml of dormicum (i.p.) and 0.04ml of atropin (s.c.) per kg bodyweight. The rats were checked for general condition, clinical signs and behavior twice daily on weekdays or once daily on weekends. The bodyweights of the rats were recorded initially and three times each week during the recovery period. At each weighing, the rats were handled and carefully examined. Approximately three weeks after recovery from cannulation, the rats were dosed orally with varying doses of the Bt proteins or reference compounds dissolved in either 5% BSA or 5% aqueous tomato extract as a vehicle. Blood samples were collected in heparin treated tubes from the vena porta at -25 hours, +0.05, +0.5, +1.0, +2, +3, +4, +6, +7 and +7.5 hours after dosing (approximately 0.2ml per sample time). The levels of Bt in the blood were analyzed by an ELISA. The dosing solutions were prepared freshly for each administration. Matrix influences were examined by the use of a 10% (w/v) suspension of lyophilized transgenic or non-transgenic tomato tissue in 0.9% saline immediately before use. These suspensions were neutralized with NaOH, clarified by centrifugation (10,000 x g for 5 min.) then diluted 1:1 with a Bt protein stock solution. The vehicles used for the controls were adjusted to have similar protein and buffer compositions. There were nine experiments described in the

accompanying table all of which were dosed with 5 ml of test solution containing from 2.6 to 298mg/kg of Cry9C.

Experiment	Annex 1	Cry9C Bt protein dose (mg/kg)	No. of male rats	ml/animal
1	4	2.6 ± 0.1	6	5.0
2	5	41.9 ± 1.8	4	5.0
3	6	272.0 ± 27.0	9	5.0
4	7	298 ± 6.0	4	5.0
5	8	198 ± 2.9	4	5.0
6	9	234.7 ± 8.1	4	5.0+TM
7	10	229.0 ± 11.6	4	5.0+Bt-TM
8	11	10.6 ± 0.7*	4	5.0
9	12	10.6 ± 0.3*	4	5.0+TM

\*Cry1Ab5; TM= tomato matrix San Marzano TL-0001; Bt-TM=Bt-tomato matrix San Marzano RLE15-0001

The validity of the animal model for bioavailability was checked using oral dose of the following substances:  $^{14}\text{C}$ -PEG, a marker of paracellular transport at 19.3-20.3  $\mu\text{Ci}/\text{rat}$  and  $^3\text{H}$ -propranolol, a marker of transcellular transport at 29.2-30.7  $\mu\text{Ci}/\text{rat}$ .

**Examination of gastro-intestinal tract tissue.** At terminal sacrifice, samples were taken from the GI tract for sectioning and histological examination (including immuno-staining). The following tissues were examined: midsegment stomach (between fore and glandular stomach), a duodenal segment (3cm distal from pylorus), a jejunal segment (3cm distal from the ilexura duodenojejunalis 40cm from the pylorus), an ileosegment (3cm proximal from the ileocaecalis transition), a midsegment of the caecum and a colonic segment (3cm distal from the valva ileocaecalis). The gut tissue samples were preserved in Bouin Hollande's 10% sublimate fixative, embedded in paraffin wax, sectioned at 5 $\mu\text{m}$  and stained with haematoxylin/eosin. Gut tissue of Lys mutant Cry9C treated rats were also stained immunocytochemically to determine binding of the Cry9C protein.

Gastric and luminal debris was collected from the remaining parts of the GI tract after opening these fragments longitudinally. Aliquots of chyme were immediately diluted 1:1 in PBS (pH8.5) with 1.5mM PMSF and 2.0mM EDTA and stored at -80°C until analysis. The procedure was validated by Bt protein spiking in the range of 0.05 to 0.5 ng/mg luminal debris. Feces were pooled over 24 hours and stored frozen at -80°C until analysis. Aliquots of frozen feces were diluted 1:1 in 50mM TrisHCl (pH7.3) containing 1.0mM PMSF and 2.0mM EDTA, extracted for one hour on ice and the supernatant diluted 1:1 with 10.0mM TrisHCl (pH8.0) containing 1.0mM EDTA, 20% glycerol, 5%

SDS, 2% dithiothreitol and 0.2% bromophenol blue. This solution was heated to 100°C for 5 minutes and stored at -40°C until western blotting.

Blood samples from the labeled PEG and propranolol treatments were examined by liquid scintillation counting after placing 150µl of serum in 3ml of PicoAqua and shaking until clear. Fifty mg samples of feces or chyme were diluted in 0.5ml of water, rehydrated for 0.5 hours, suspended in 3.0ml of Soluene 350, swirled and incubated at 60° overnight. After this incubation, 0.5ml of 2-propanol was added and incubated for 5 minutes at 60°C, then 0.2ml of 30% hydrogen peroxide were added three times and incubated for 2 hours. After cooling to room temperature, 15 ml of Hionic fluor was added to the samples and they were counted.

**Enzyme linked immunosorbent assay (ELISA)** An indirect double antibody sandwich assay was used to detect levels of the Lys mutant Cry9C in blood. The ELISA was performed according to SOP PGS-9D/1 which was not included. The coating/capture antibody was RÓCry9C followed by a 1:1000 dilution of GÓCry9C as the secondary antibody and a 1:10,000 dilution of RÓGIgG-HRP conjugate as the detection antibody. The sensitivity of the ELISA technique was checked by running spiked samples of blood and chyme in sixfold dilutions from 1 to 100 ngBt protein/ml sample.

**SDS-PAGE** was performed using a Pharmacia Multifor II horizontal electrophoresis apparatus with ExcelGel homogenous 12.5% SDS gels. The protein samples to be analyzed were prepared by 1:1 dilution with Laemmli sample buffer with dithiothreitol and boiled at 100°C for 5 minutes.

**Iso-electric focusing** was performed on a Pharmacia Multifor II horizontal electrophoresis apparatus with ready to use Ampholyte buffer strips (pH 3.0-10.0). The samples were prepared according to manufacturer's manual which was not included in the submission.

**2D-gel electrophoresis** was performed on a Pharmacia Multifor II horizontal electrophoresis apparatus with ready to use Ampholyte buffer strips (pH 3.0-10.0) and ExcelGel homogenous 12.5% SDS gels. The samples were prepared according to manufacturer's manual which was not included in the submission.

**Capillary zone electrophoresis** was done with a Spectra Phoresis 1000 (Thermo Separation Products) equipped with a fused silica capillary (70 cm x 75 µm, Thermo Separation Products) or a hydrophilic coated capillary (70 cm x 75 µm) CElect P175 (Supelco). The capillary was cleaned by rinsing with 0.1N NaOH at the beginning of the day and allowed to equilibrate with a running buffer rinse for about 2-5 minutes prior to the first sample. The buffer reservoir at the cathode was filled with the same running buffer. The 20nl samples were hydrodynamic (in vacuo) for 5 seconds during injection and introduced at the anode. The voltage across the capillary was 15kV.

**Protein band visualization** was accomplished for any of the above protein separation techniques by either Coomassie brilliant blue G-250 staining or by silver staining.

**Western blotting** was performed on the Bt proteins or their proteolytic fragments after they were first separated by SDS-PAGE. The proteins in the SDS-PAGE gels were transferred to either nitrocellulose or Hyperbond-H membranes by semi-dry blotting between two graphite plates. The

membranes were blocked for non-specific binding of antibodies with BSA then probed for the presence of Cry proteins with a 1:1000 dilution of Cry specific rabbit polyclonal antibodies. After rinsing, any bound specific rabbit antibody was detected using a goat anti-rabbit IgG antibody conjugate with alkaline phosphatase. The bound antibody complexes were visualized by the chromogenic substrate BCIP/NBT. Protein recovery and extent of degradation was determined by scanning band intensity in a Scanmaster desk-top flat bed scanner compared to reference bands as analyzed by Diversity One software. For the less intense anti-cry protein bands, ECL methodology (Amersham) was used to boost sensitivity.

**Receptor binding assay** The *in vitro* binding of the Lys mutant Cry9C protein to intestinal tissues of rats or rhesus monkeys was done by an immunocytological method. Basically deparaffinated, rehydrated sections were incubated with Lugol solution and 5% sodium thiosulphate. These sections were then rinsed and incubated with methanol and 0.6% hydrogen peroxide to inactivate the endogenous peroxidases then the rinsed sections were incubated with a Cry9C solution (50µg/ml) overnight at 4°C. Any bound Cry9C was detected by binding of a rabbit polyclonal anti-Cry9C antibody and visualized with a sheep anti-rabbit antibody conjugate with HRP (1 hour incubation at room temperature). The bound HRP conjugate was visualized by the reaction of DAB and the other cells were stained with heamalin, dehydrated and covered with mounting medium. Gut membranes of *Maduca sexta* and *Ostrina nubilalis* were prepared to provide positive binding controls and specificity of the assay was confirmed by exclusion of the primary or secondary antibody or inclusion of a rabbit antibody to BSA.

**Osmotic resistance assay** was done according to the protocol described in Noteborn *et al.*, (1995, ACS symposium Series 605, p. 134-147). This reference was not provided.

## RESULTS AND DISCUSSION

The cited data from the submission is included in an appendix to this review.

### **Characterization of test material**

All three batches of Lys mutant Cry9C were shown by SDS-PAGE and western blot to be substantially made up of Cry9C protein compared to the 100% standard provided by PGS. Greater than 90% of each preparation had the 68 kDa band as the major component with the majority of the remaining contaminant being the 55 kDa degradation form. Other minor bands were seen but there amounts or molecular weights were not stated nor were the gels used for justifying these claims presented. The capillary zone electrophoresis results indicated similar findings with there being two major peaks, 85-90% being the 68kDa form and 55kDa form being the other peak.

The batches were said to vary from each other and the supplied standard but no attempt was made to qualify the differences and none of these variations were claimed to alter the statement of > 90% Cry9C purity given above. The stated sensitivity of the gel assays was 1-2ng protein/band. The Cry9C protein was soluble to 18.0mg/ml in a buffer above pH 8.0. The ELISA results were used to calculate all the dose amounts even though it was stated that this assay consistently overestimated the protein content compared to conventional protein assays corrected for purity by about 5%.

### **Stability to *in vitro* digestion and heat**

The three batches of Lys mutant Cry9C either from *E. coli* (batches I & II) or *B. thuringiensis* (batch III) were shown to be substantially resistant to degradation after 2 hours by either SGF or SIF. The samples were taken from SGF or SIF incubation solution and loaded at 19.5µg Cry9C/lane on the gels. In SGF there was an initial decrease of 15-25% compared to the Cry9C buffer control after 15 minutes incubation which did not decrease further after 2 hour's total SGF incubation. There was no decrease at all in the Cry9C concentration resulting from SIF incubation. Two silver stained gels were presented for each of the *in vitro* digestions. In one of the SGF gels (figure 3) it is curious to note that no 55kDa form of Cry9C is present in the gel although this is stated as always being a major contaminant.

When the Cry9C samples were subject to heat (90°C) for extended periods the protein appear to be stable as the protein banding pattern in SDS-PAGE gels is unaltered. The presence of a tomato matrix does not appear to affect this heat stability. However, the company has presented an isoelectric focusing gel which purports to show a loss of detectable protein forms after 30 to 60 minutes. They state that this contradictory data is due to the spontaneous aggregation of Cry9C into higher molecular weight forms. None of these higher molecular weight aggregate forms are seen in the SDS-PAGE gels. It is unclear how SDS-PAGE alone would detect heat stability since all samples are routinely heated prior to gel analysis. Probably more appropriate endpoint to measure thermostability would be an insect bioassay which would at least detect degradation from the active, toxic 68kDa to the non-toxic 55kDa form.

#### **Stability to *in vivo* digestion**

Nine male Wistar rats were dosed with 83 mg of Cry9C by oral gavage equivalent to a dose of approximately 283±20mg/kg bodyweight. Eight hours after dosing, gastric and luminal debris were collected, separated by SDS-PAGE and the molecular weight and stability were examined by immunoblot (figure 13). While the gel only includes 4 rats, the results indicate that the Cry9C protein is degraded to the 55kDa form over time and that is the major immunoreactive band found in the caecum and colon.

#### **Bioavailability in the rat**

Data using radiolabelled PEG and propranolol were used to validate that artificial substances can be tracked in rats and mimic the absorption of food proteins. Also assumptions about bioavailability between a large molecular weight compound (not readily absorbed) and a small model solute (rapidly absorbed as a first order rate process) were valid. The data show that GI passage is about 24 hours and that a total of approximately 30% of the model solute propranolol is bioavailable whereas about 0.03% of the large molecular weight compound PEG is bioavailable. No indication of the amount of the PEG or propranolol administered was given although the radioactivity counts were well described.

The data for bioavailability of Cry9C protein was monitored by the indirect double antibody sandwich ELISA previously described which had a detection limit of 2.0 ng Cry9C/ml in serum with a recovery rate of 85±5%. Previous work with Cry1Ab5 is also presented and that ELISA had a detection limit of 5.0 ng Cry1Ab5/ml with a recovery rate of 55±5%. The animals were dosed with a range of Cry9C from 2.6 to 298 mg/kg bodyweight by gastric intubation. All the dosed animals

appeared and behaved normally, survived to sacrifice and displayed no treatment related adverse effects.

No ELISA positive Cry9C protein or fragments were detected in blood samples taken from rats at the 2.6mg/kg dose. At the higher dose rates, Cry9C ELISA values were positive giving a calculated value of between 5 and 15 ng Cry9C/ml in serum. The absorption rate was not affected by dose amount or presence of a food matrix. The percentage of bioavailable Cry9C was calculated to range from 0.0006% (42mg/kg) to 0.0002% (298mg/kg) of the dose administered.

More importantly, an SDS-PAGE/western blot confirmation of the sera that were ELISA positive for the presence of Cry9C protein showed that only one of the rats had serum with confirmed Cry9C protein. This rat (#548) had a faint Cry9C positive band, all the other sera had no detectable Cry9C reactivities. No interfering compounds were found in rat serum or BSA. The positive signals were attributed to cross-reaction with Cry9C like components present in both control and Bt-treated animals since there were no significant differences between the 2-D electrophoresis or ECL results. This data indicating cross reaction was not presented.

#### **Immunocytochemical analysis and hemolytic effects**

Intestinal tissue from rats, rhesus monkeys and humans was used to examine the binding of Cry9C to the intestinal membranes in tissue sections. A rabbit polyclonal IgG antibody was raised against Cry9C as the detection reagent. A positive Cry9C binding control using sections of brush border epithelial cells from *Ostrinia nubilalis* or *Manduca sexta* was also examined. No positive specific binding was reported for the non-arthropod species tested. However, a high degree of nonspecific antibody binding to the tissues from *Ostrinia nubilalis* or *Manduca sexta* even in the absence of Cry9C was reported. This background binding was difficult to reduce but the best positive reaction was obtained with *Ostrinia nubilalis* tissues using 3% BSA as a blocking agent and increasing the Cry9C incubations to 15 minutes.

Examination of the GI tissue samples from the nine rats in the high dose Cry9C group revealed no Cry9C binding to any tissue of the GI tract. The villi and individual enterocytes lining the crypts of the small and large intestines were normal compared to these same tissue sections in the untreated controls.

Human red blood cells were examined for any hemolytic effects after incubation with Cry9C by monitoring for osmotic fragility spectroscopically. No hemolysis was indicated by the assay.

No appendix 5 is included in the submission although many of the techniques refer to it.

# APPENDIX TO BIOAVAILABILITY

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ANNEX 1 - Table 2

LYS MUTANT CRY9C ORAL STABILITY TO DIGESTION in vitro  
 RIKILT-DLO  
 Study: Digestibility *in vitro* - Simulated Intestinal Fluid (SIF)

Table 2 Stability as percentage (%) of parent band (native protein)<sup>1</sup>

Time (min)		Protein				
		Cry9C	Cry1Ab5	CryIIIb	NPTII	PAT
0	Mean	100	100	100		0
2	Mean	100	100	100		10
	SD					7
	n	4	4	4		4
5	Mean	100	100	100		3
	SD					1
	n	4	4	4		4
15	Mean	100	100	1010		0
	SD					
	n	4	4	4		4
30	Mean	100	100	100		0
	SD					
	n	4	4	4		4
60	Mean	100	100	100		0
	SD					
	n	4	4	4		4
120	Mean	100	100	100		0
	SD					
	n	4	4	4		4

Statistics:

ANOVA: \* P < 0.05 \*\* P < 0.01

<sup>1</sup>: western blots measured by scanning densitometer

The absence of a percentage indicates that the process specified was not recorded at that time point.

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ANNEX 1 - Table 1

LYS MUTANT CRY9C ORAL STABILITY TO DIGESTION *in vitro*  
 RIKILT-DLO  
 Study: Digestibility *in vitro* - Simulated Gastric Fluid (SGF)

Time (min)		Protein				
		Cry9C	Cry1Ab5	CryIIIb	NPTII	PAT
0	Mean	100	100	100	0	0
2	Mean	96	41	13	9	50
	SD	4	9	7	3	10
	n	4	4	4	4	4
5	Mean	86		0	0	0
	SD	3		4	4	4
	n	4				
15	Mean	82	21	0	0	0
	SD	5	5	4	4	4
	n	4	4	4	4	4
30	Mean	78	21	0	0	0
	SD	6	8	4	4	4
	n	4	4	4	4	4
60	Mean	77	11	0	0	0
	SD	3	6	4	4	4
	n	4	4	4	4	4
120	Mean	78	9	0	0	0
	SD	5	4	4	4	4
	n	4	4	4	4	4

## Statistics:

ANOVA: \*  $P < 0.05$  \*\*  $P < 0.01$ 

†: western blots measured by scanning densitometer

The absence of a percentage indicates that the process specified was not recorded at that time point.

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## ANNEX 1 - Table 4

LYS MUTANT CRY9C ORAL BIOAVAILABILITY  
RIKILT-DLO

RAT

Study: Wistar (6 males) - bleeding from portal vein canula

Dose: 1.0 mg Lys mutant Cry9C/rat/5.0 ml of 0.9% NaCl (pH 7.2) containing 5% (w/v) BSA

Time (hrs)	Concentration (ng/ml of plasma) in the various animals					
	521 (418 g)	522 (391 g)	523 (368 g)	524 (407 g)	525 (419 g)	528 (357 g)
0 Mean 0 SD	0	0	0	0	0	0
0.05 Mean SD	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0.5 Mean SD	n.d.	n.d.	n.d.	n.d.		n.d.
1.0 Mean SD	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2.0 Mean SD	n.d.	n.d.	n.d.			n.d.
3.0 Mean SD	n.d.	n.d.	n.d.	n.d.	0.81	n.d.
4.0 Mean SD	n.d.	n.d.	0.8			n.d.
6.0 Mean SD	n.d.	n.d.	0.88	n.d.		n.d.
7.0 Mean SD	n.d.	n.d.	1.14	n.d.	1.38	n.d.
7.5 Mean SD						
Dose (mg/kg)	2.4	2.6	2.7	2.5	2.4	2.8
Mean	2.57					
SD	0.14					

Statistics:

ANOVA: \* P &lt; 0.05 \*\* P &lt; 0.01

n.d.: not detectable

The absence of a concentration indicates that the blood sample specified was not taken.

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ANNEX 1 - Table 5

LYS MUTANT CRY9C ORAL BIOAVAILABILITY RAT  
RIKILT-DLO

Study: Wistar (4 males) - bleeding from portal vein canula

Dose: 13 mg Lys mutant Cry9C/rat/5.0 ml of 20 mM TrisHCl/0.5 mM NaEDTA (pH 8.6) containing 5% (w/v) BSA

Table 5 Plasma levels of Cry9C measured by DS-ELISA						
Time (hrs)			Concentration (ng/ml of plasma) in the various animals			
			569 (331 g)	573 (309 g)	575 (317 g)	576 (289 g)
0	Mean SD	0	0	0	0	0
0.05	Mean SD	3.2 1.4	3.48	2.32	5.07	1.81
0.5	Mean SD	5.2 0.9		3.94	5.61	6.03
1.0	Mean SD	5.8 1.5		4.3	7.24	
2.0	Mean SD	6.7 2.2	7.87	4.23	5.61	9.1
3.0	Mean SD	3.9 0.9		2.93	4.78	
4.0	Mean SD	4.7 2.8	4.56	0.85	4.65	8.82
6.0	Mean SD					
7.0	Mean SD	5.2 4.2	3.47	1.31	3.74	12.27
7.5	Mean SD	8.0 9.5	3.67	1.74	2.03	24.39
	Dose (mg/kg)		39.3	42.1	41.0	45.0
	Mean SD	41.9 1.8				

Statistics:

ANOVA: \* P < 0.05 \*\* P < 0.01

The absence of a concentration indicates that the blood sample specified was not taken.

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ANNEX 1 - Table 6

LYS MUTANT CRY9C ORAL BIOAVAILABILITY RAT  
RIKILT-DLO

Study: Wistar (9 males) - bleeding from portal vein canula

Dose: 83 mg Lys mutant Cry9C/rat/5.0 ml of 20 mM TrisHCl/0.5 mM NaEDTA (pH 8.6) containing 5% (w/v) BSA

Time (hrs)	Concentration (ng/ml of plasma) in the various animals									
	547 (295 g)	548 (287 g)	549 (275 g)	551 (262 g)	556 (352 g)	558 (342 g)	561 (340 g)	562 (327 g)	550 (293 g)	
0 Mean	0	0	0	0	0	0	0	0	0	
0.05 Mean	0.88	0	0	0	0.8	0	0	0.9	0	
SD										
0.5 Mean	23.7	74.6	21.2	16.4	1.2	13.5	0	0.8	0	
SD										
1.0 Mean	2.1	6.5		22.0	3.5	17.7	0.7	1.2	1.6	
SD										
2.0 Mean	4.7	3.3		27.7	7.4	20.9	6.8	7.0	4.8	
SD										
3.0 Mean	4.0	3.7		29.7	7.6	20.4	31.2	27.8	5.0	
SD										
4.0 Mean	12.5	2.4	12.7	31.4	15.6	20.2		23.4	5.4	
SD										
6.0 Mean	3.4	1.2		23.7	14.8	34.6		33.3	3.4	
SD										
7.0 Mean	11.7	6.7		37.1	12.7	21.0	5.6	12.4	12.3	
SD										
7.5 Mean	3.73	4.3	3.4	28.6	10.0	17.2	5.1	10.4	9.2	
SD										
Dose (mg/kg)	281.4	289.2	301.8	316.8	235.8	242.7	253.8	244.1	283.3	
Mean	272.1									
SD	27.3									

Statistics: ANOVA \* P < 0.05 \*\* P < 0.01

Rat no. 555 (334 g) and 560 (342 g) were dosed with vehicle only and served as DS-ELISA controls.

The absence of a concentration indicates that the blood sample specified was not taken.

24E

036

## ANNEX 1 - Table 7

LYS MUTANT CRY9C ORAL BIOAVAILABILITY  
RIKILT-DLO

RAT

Study: Wistar (4 males) - bleeding from portal vein canula

Dose: 91 mg Lys mutant Cry9C/rat/5.0 ml of 20 mM TrisHCl/0.5 mM NaEDTA (pH 8.6) containing 5% (w/v) BSA

Time (hrs)	Concentration (ng/ml of plasma) in the various animals					
			564 (303 g)	565 (310 g)	568 (310 g)	574 (298 g)
0	Mean	0	0	0	0	0
	SD					
0.05	Mean	1.60	1.84	1.55	1.14	1.85
	SD	0.3				
0.5	Mean	5.49	5.92	6.55	4.80	6.03
	SD	0.6				
1.0	Mean	9.97	7.17	19.83	6.99	5.93
	SD	6.5				
2.0	Mean	7.51	8.08	13.83	6.01	5.1
	SD	2.1				
3.0	Mean	6.32	7.44	15.07	4.39	2.2
	SD	2.7				
4.0	Mean	6.18	6.92	15.70	3.75	3.82
	SD	5.8				
6.0	Mean	4.01	2.84	10.28	2.93	2.27
	SD	1.6				
7.0	Mean	2.35	1.61	8.31	0.89	1.41
	SD	2.1				
7.5	Mean	6.6	3.02	9.4	8.5	2.0
	SD	2.7				
	Dose (mg/kg)		300.3	293.5	293.5	305.4
	Mean	298.2				
	SD	5.7				

Statistics: ANOVA \* P &lt; 0.05 \*\* P &lt; 0.01

The absence of a concentration indicates that the blood sample specified was not taken.

24F  
037

hA

## ANNEX 1 - Table 8

LYS MUTANT CRY9C ORAL BIOAVAILABILITY  
RIKILT-DLO

RAT

Study: Wistar (4 males) - bleeding from portal vein canula

Dose: 60 mg Lys mutant Cry9C/rat/5.0 ml of 20 mM TrisHCl/0.5 mM NaEDTA (pH 8.6) containing 5% (w/v) BSA

Table 8 Plasma levels of Cry9C measured by DS-ELISA

Time (hrs)	Concentration (ng/ml of plasma) in the various animals			
	530 (301 g)	531 (296 g)	532 (306 g)	534 (307 g)
0 Mean 0 SD	0	0	0	0
0.05 Mean 1.6 SD 0.4	1.69	1.12	2.32	1.23
0.5 Mean 5.8 SD 0.9	6.82	6.45	4.98	4.78
1.0 Mean 7.2 SD 1.5	6.91	7.76	9.09	5.08
2.0 Mean 8.7 SD 1.8	10.77	10.02	7.56	6.34
3.0 Mean 7.9 SD 1.7	8.9	9.72	8.06	5.1
4.0 Mean 5.5 SD 1.8	6.45	7.13	5.91	2.58
6.0 Mean SD				
7.0 Mean SD				
7.5 Mean 2.1 SD 1.0	1.23	3.23	2.92	1.1
Dose (mg/kg) Mean 198.4 SD 2.9	199.3	202.7	196.1	195.4

## Statistics:

ANOVA: \* P &lt; 0.05 \*\* P &lt; 0.01

The absence of a concentration indicates that the blood sample specified was not taken.

246

038

## ANNEX 1 - Table 9

LYS MUTANT CRY9C ORAL BIOAVAILABILITY RAT  
 RIKILT-DLO

Study: Wistar (4 males) - bleeding from portal vein canula

Dose: 71.6 mg Lys mutant Cry9C/rat/5.0 ml of 20 mM TrisHCl/0.5 mM NaEDTA (pH 8.6) containing 5% (w/v) tomato matrix (variety: TL-0001)

Table 9			Plasma levels of Cry9C measured by DS-ELISA			
Time (hrs)			Concentration (ng/ml of plasma) in the various animals			
			563 (310 g)	567 (316 g)	570 (311 g)	577 (285 g)
0	Mean SD	0	0	0	0	0
0.05	Mean SD	2.5 1.6	1.46	0.92	5.02	2.69
0.5	Mean SD	6.2 0.5	5.56	6.12	6.96	6.23
1.0	Mean SD	8.7 3.5	5.19	9.85	13.95	5.91
2.0	Mean SD	10.9 3.3	7.07	14.56	8.14	9.1
3.0	Mean SD	8.9 1.7	6.38	10.72	8.43	13.77
4.0	Mean SD	6.0 1.5	3.69	7.45	5.62	10.04
6.0	Mean SD					
7.0	Mean SD					
7.5	Mean SD	8.5 4.0	3.48	14.29	9.65	7.13
	Dose (mg/kg)		230.9	226.5	229.5	251.2
	Mean	234.7				
	SD	8.1				

Statistics:

ANOVA: \* P < 0.05 \*\* P < 0.01

The absence of a concentration indicates that the blood sample specified was not taken.

24H  
033

ANNEX 1 - Table 10

LYS MUTANT CRY9C ORAL BIOAVAILABILITY RAT  
RIKILT-DLO

Study: Wistar (4 males) - bleeding from portal vein canula

Dose: 69.6 mg Lys mutant Cry9C/rat/5.0 ml of 20 mM TrisHCl/0.5 mM NaEDTA (pH 8.6) containing 5% (w/v) Bt-tomato matrix (variety: RLE15-0001)

Table 10			Plasma levels of Cry9C measured by DS-ELISA			
Time (hrs)			Concentration (ng/ml of plasma) in the various animals			
			566 (311 g)	571 (276 g)	572 (316 g)	578 (317 g)
0	Mean SD	0	0	0	0	0
0.05	Mean SD	1.8 0.8	1.00	2.15	2.85	1.03
0.5	Mean SD	6.2 2.2	4.62	9.08	3.55	7.62
1.0	Mean SD	8.6 1.8	7.23	11.72	8.3	7.3
2.0	Mean SD	7.1 1.4	8.20	8.64	6.7	5.0
3.0	Mean SD	6.6 2.5	10.73	6.6	4.8	4.4
4.0	Mean SD	4.0 1.9	6.93	4.2	1.9	3.1
6.0	Mean SD					
7.0	Mean SD	5.3 2.1	7.4	7.3	2.4	4.1
7.5	Mean SD	7.9 4.7	9.5	14.6	2.1	5.2
	Dose (mg/kg) Mean SD		223.8	252.2	220.3	219.6

Statistics:

ANOVA: \* P < 0.05 \*\* P < 0.01

The absence of a concentration indicates that the blood sample specified was not taken.

241  
040

ANNEX 1 - Table 11

CRY1Ab5 ORAL BIOAVAILABILITY RAT  
 RIKILT-DLO  
 Study: Wistar (4 males) - bleeding from portal vein canula  
 Dose: 3.5 mg Cry1Ab5/rat/5.0 ml of 50 mM Na<sub>2</sub>CO<sub>3</sub>/0.2 mM NaCl (pH 7.2) containing 5% (w/v)  
 BSA

Time (hrs)		Concentration (ng/ml of plasma) in the various animals			
		510 (300 g)	514 (332 g)	516 (342 g)	517 (356 g)
0	Mean SD	0	0	0	0
0.05	Mean SD	n.d.	n.d.	n.d.	n.d.
0.5	Mean SD	n.d.	n.d.	n.d.	n.d.
1.0	Mean SD	n.d.	n.d.	n.d.	n.d.
2.0	Mean SD	n.d.	n.d.	n.d.	n.d.
3.0	Mean SD	n.d.	n.d.	n.d.	n.d.
4.0	Mean SD	n.d.	n.d.	n.d.	n.d.
6.0	Mean SD	n.d.			
7.0	Mean SD	n.d.			
24	Mean SD	n.d.	n.d.	n.d.	n.d.
	Dose (mg/kg)	11.7	10.5	10.2	9.8
	Mean	10.6			
	SD	0.7			

Statistics: ANOVA  $P < 0.05$  \*\*  $P < 0.01$

n.d.: not detectable

The absence of a concentration indicates that the blood sample specified was not taken.

24 J  
041

## ANNEX 1 - Table 12

CRY1Ab5 ORAL BIOAVAILABILITY RAT  
RIKILT-DLO

Study: Wistar (4 males) - bleeding from portal vein canula

Dose: 3.5 mg Cry1Ab5/ra/5.0 ml of 50 mM Na<sub>2</sub>CO<sub>3</sub>/0.2 mM NaCl (pH 7.2) containing 5% (w/v) tomato matrix (variety: TL-0001)

Time (hrs)	Concentration (ng/ml of plasma) in the various animals			
	511 (322 g)	515 (342 g)	518 (319 g)	519 (341 g)
0 Mean SD	0	0	0	0
0.05 Mean SD	n.d.	n.d.	n.d.	n.d.
0.5 Mean SD	n.d.	n.d.	n.d.	n.d.
1.0 Mean SD	n.d.	n.d.	n.d.	n.d.
2.0 Mean SD	n.d.	n.d.	n.d.	n.d.
3.0 Mean SD	n.d.	n.d.	n.d.	n.d.
4.0 Mean SD	n.d.	n.d.	n.d.	n.d.
6.0 Mean SD	n.d.	n.d.	n.d.	n.d.
7.0 Mean SD				
24 Mean SD	n.d.	n.d.	n.d.	n.d.
Dose (mg/kg)	10.9	10.2	11.0	10.3
Mean	10.6			
SD	0.3			

Statistics: ANOVA \* P &lt; 0.05 \*\* P &lt; 0.01

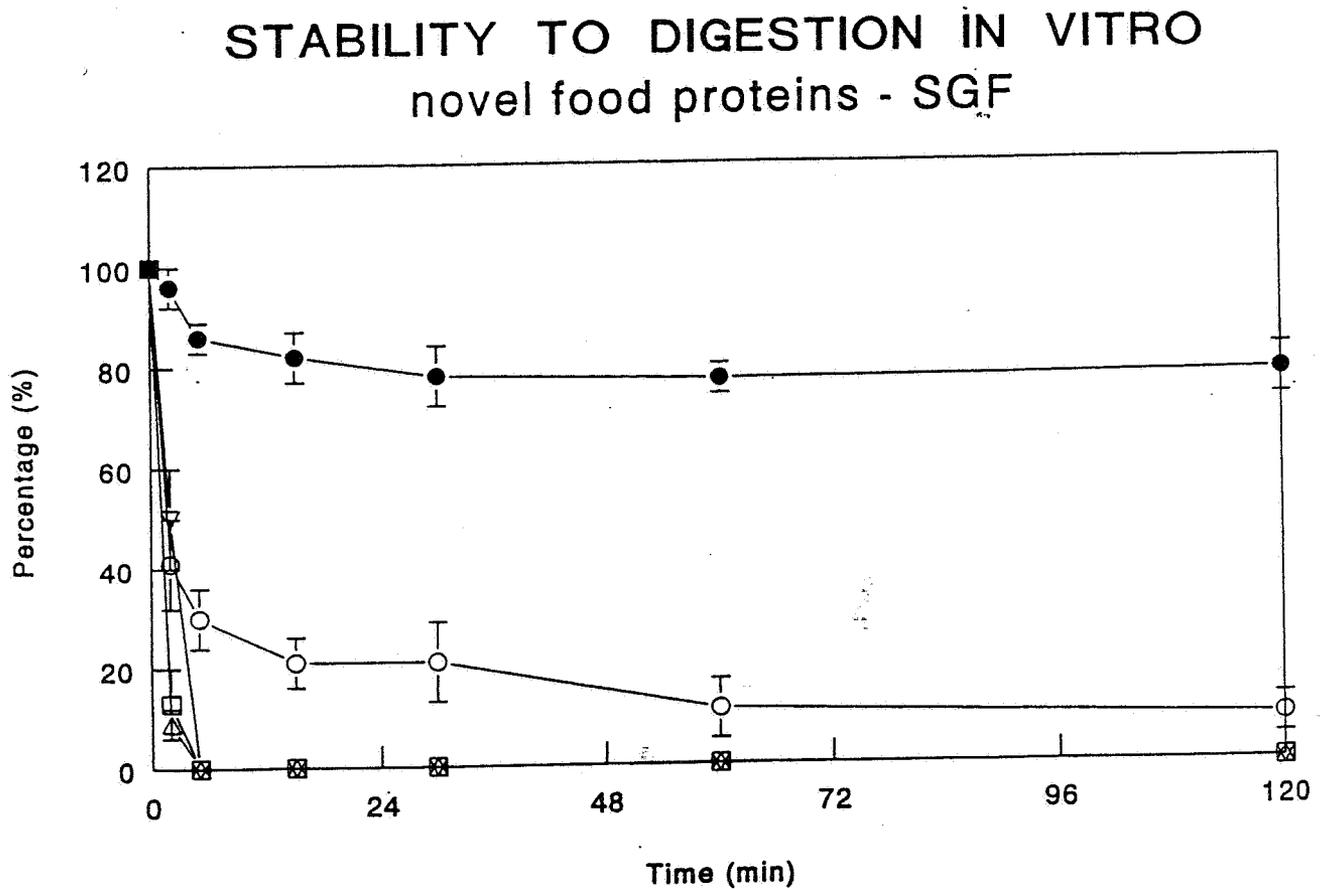
n.d.: not detectable

The absence of a concentration indicates that the blood sample specified was not taken.

24 K

042

FIGURE 1



**Figure 1.** Representative graph showing the stability of the Lys mutant Cry9C protein to digestion in vitro under simulated human gastric conditions (SGF). The rate of stability of the Cry9C protein was analyzed by scanning of the 1-D lanes with a Desk-Top flat bed densitometer and estimated by integrating the optical densities of matched Cry9C protein bands in the different 1-D lanes (i.e. SDS-PAGE and Western blots) based on a calibration curve and relative (%) to the density of unincubated gastric buffer (pH 2.0) without pepsin but with the Lys mutant Cry9C protein. The rate of stability of the Lys mutant Cry9C protein (closed circles) was also compared with that of the Cry1Ab5 protein (open circles), the CryIIIb protein (open squares), the NPTII enzyme (open triangles) and the PAT enzyme (open reversed triangles). Data represent mean values ( $\pm$  SD) of sixfold measurements per time point.

24 L  
047

FIGURE 2

### STABILITY TO DIGESTION IN VITRO novel food proteins - SIF

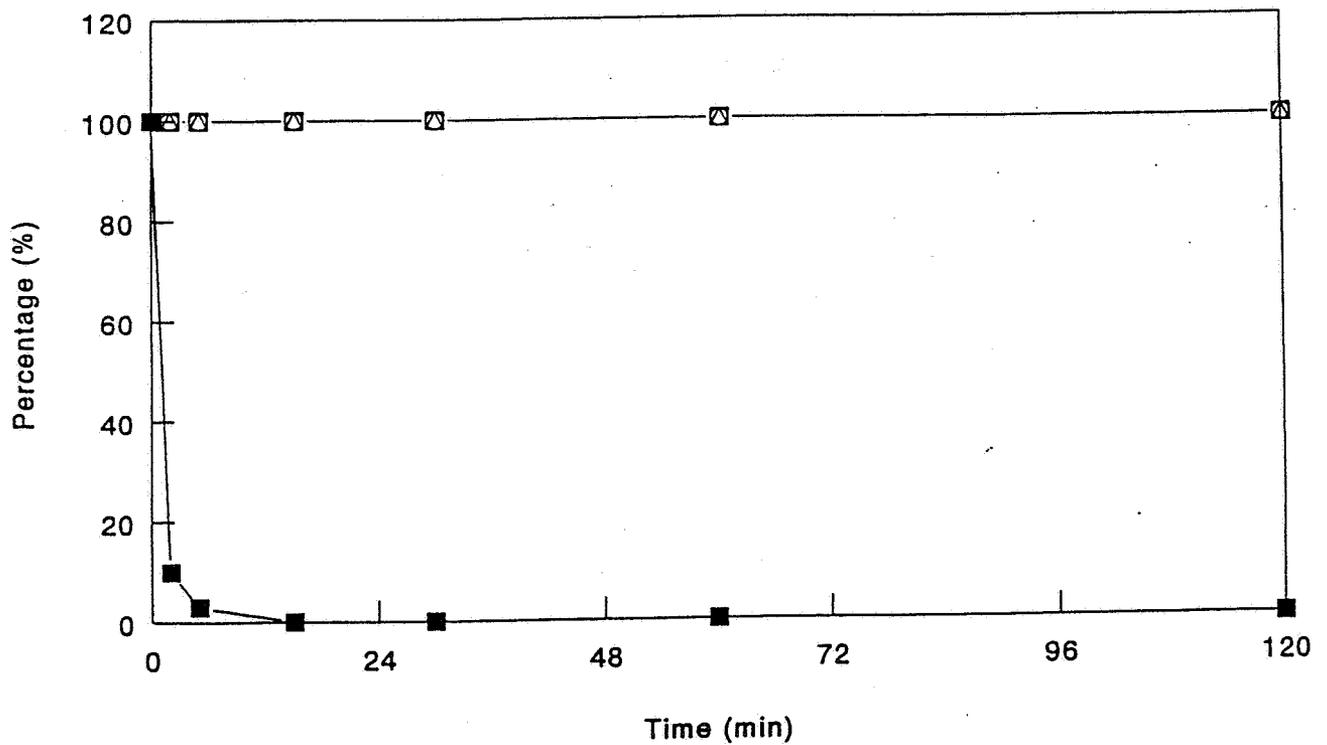


Figure 2.

Representative graph showing the stability of the Lys mutant Cry9C protein to digestion in vitro under simulated human intestinal conditions (SIF). The rate of stability of the Cry9C protein was analyzed by scanning of the 1-D lanes with a Desk-Top flat bed densitometer and estimated by integrating the optical densities of matched Cry9C protein bands in the different 1-D lanes (i.e. SDS-PAGE and Western blots) based on a calibration curve and relative (%) to the density of unincubated intestinal buffer without pancreatin but with the Lys mutant Cry9C protein (open triangles). The rate of stability of the Lys mutant Cry9C protein was compared with that of the Cry1Ab5 protein (open circles), the CryIIIb protein (open squares) and the PAT enzyme (closed squares). Data represent mean values ( $\pm$  SD) of sixfold measurements per time point.

24 M  
048

HA

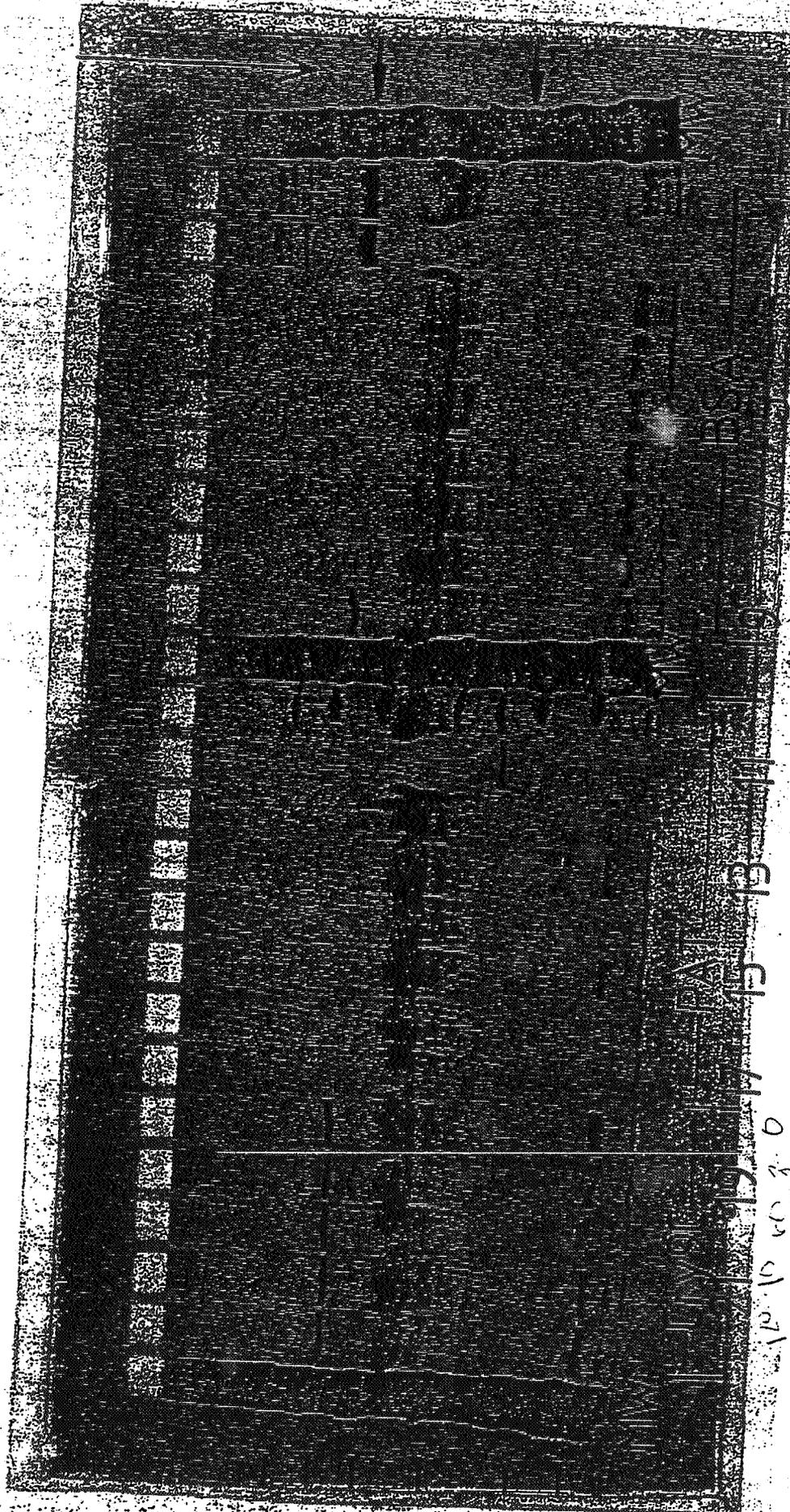


FIGURE 3

Figure 3. Profile of the degradation of Lys mutant Cry9C protein observed in simulated gastric fluid as analyzed by SDS-PAGE gel electrophoresis (SDS-PAGE, gel nr. 9611211) and stained with silver. Samples: MW, LMW marker proteins (phosphorylase B, 94 kDa; ovalbumin, 43 kDa; albumin, 67 kDa; trypsin inhibitor, 20.1 kDa; O-lactalbumin, 14.1 kDa); lanes 18 to 22, Cry9C protein (19.5 µg/lane) taken at 0 min (lane 18), at 30 min (lane 19), at 60 min (lane 20), at 90 min (lane 21) and at 120 min (lane 22) after incubation at 37°C (pH 2) in simulated gastric fluid containing pepsin (SGF); lanes 10 to 17, PAT enzyme (3 µg/lane) taken at 0, 10, 30, 60, 90 and 120 min (lane 12 to 17, respectively) after incubation in SGF, lane 10 quenched unincubated simulated gastric fluid with pepsin and PAT and lane 11, quenched unincubated simulated gastric fluid with PAT and without pepsin; lanes 1-9, BSA (7.5 µg/lane) taken at 0, 10, 30, 60, 90 and 120 min (lane 3 to 8, respectively) after incubation in SGF, lane 1 and 9 quenched (un)incubated simulated gastric fluid with pepsin and BSA and lane 2, quenched unincubated simulated gastric fluid with BSA and without pepsin.

049 24N  
 16 19 21 22  
 12 13 14 15 16 17 18 19 20 21 22

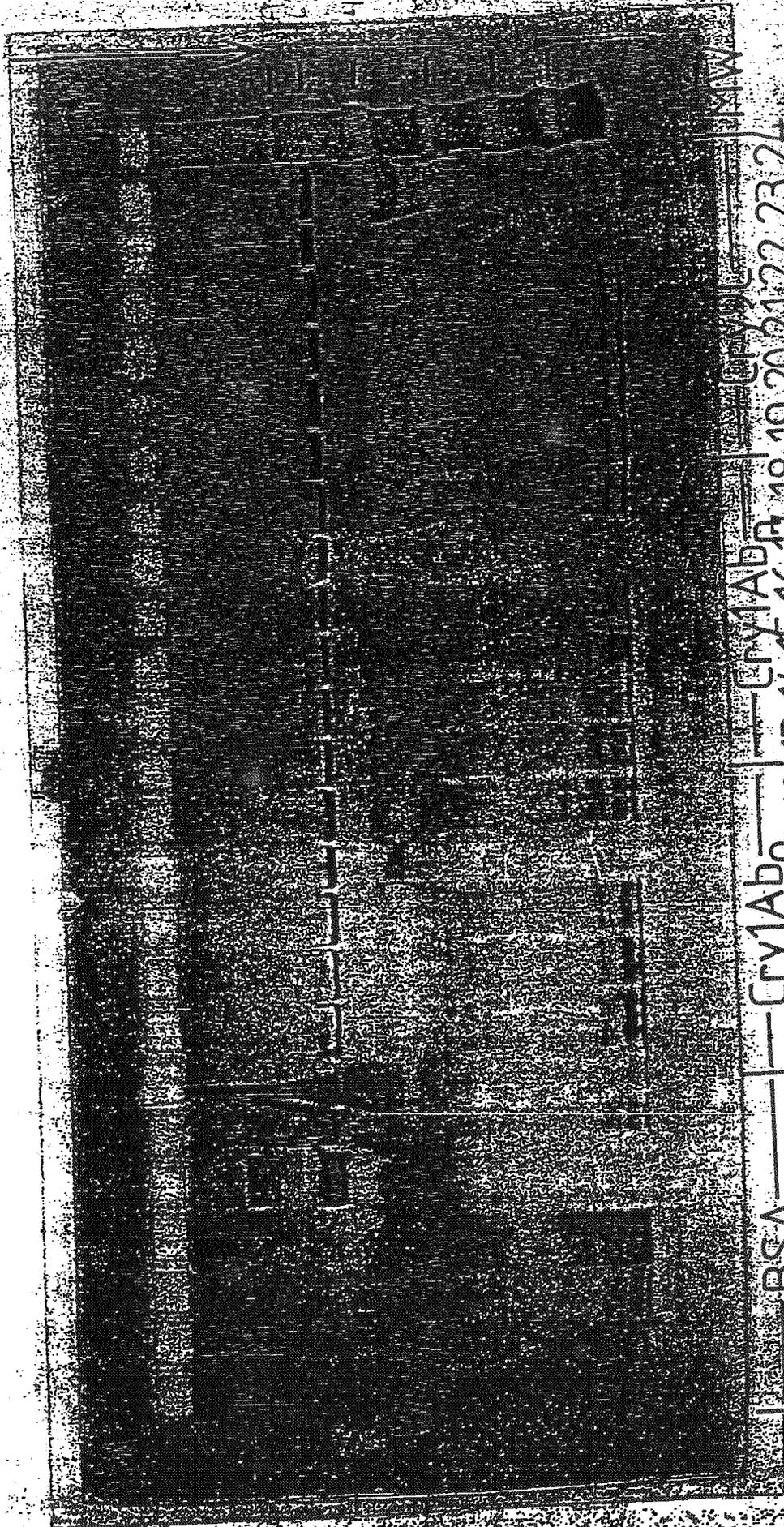


FIGURE 4

Figure 4. Profile of the degradation of Lys mutant Cry9C protein observed in simulated gastric fluid as analyzed by SDS-PAGE gel electrophoresis (SDS-PAGE, gel nr. 961210) and stained with silver. Samples: MW, LMW marker proteins (phosphorylase B, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; O-lactalbumin, 14.1 kDa); lanes 22 to 19, Cry9C protein (19.5 µg/lane) taken at 0 min (lane 22), at 30 min (lane 21), at 60 min (lane 20) and at 120 min (lane 19) after incubation at 37°C (pH 2) in simulated gastric fluid containing pepsin (SGF), lane 24 quenched unincubated simulated gastric fluid with pepsin and Cry9C and lane 23 quenched unincubated simulated gastric fluid with Cry9C and without pepsin; lanes 16 to 13, Cry1Ab5 (batch new, 22.3 µg/lane) taken at 0, 30, 60 and 120 min (lane 16 to 13, respectively) after incubation in SGF, lane 18 quenched unincubated simulated gastric fluid with pepsin and Cry1Ab5 and lane 17 quenched unincubated simulated gastric fluid with Cry1Ab5 and without pepsin; lanes 10 to 7, Cry1Ab5 (batch old, 5.3 µg/lane) taken at 0, 30, 60 and 120 min (lane 10 to 7, respectively) after incubation in SGF, lane 12 quenched unincubated simulated gastric fluid with pepsin and Cry1Ab5 and lane 11 quenched unincubated simulated gastric fluid with Cry1Ab5 and without pepsin; lane 4 to 1, BSA (7.5 µg/lane) taken at 0, 1, 5 and 10 min after incubation in SGF, lane 6 and 5 quenched (un)incubated simulated gastric fluid with pepsin and BSA.

BSA  
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24  
MW

FIGURE 5

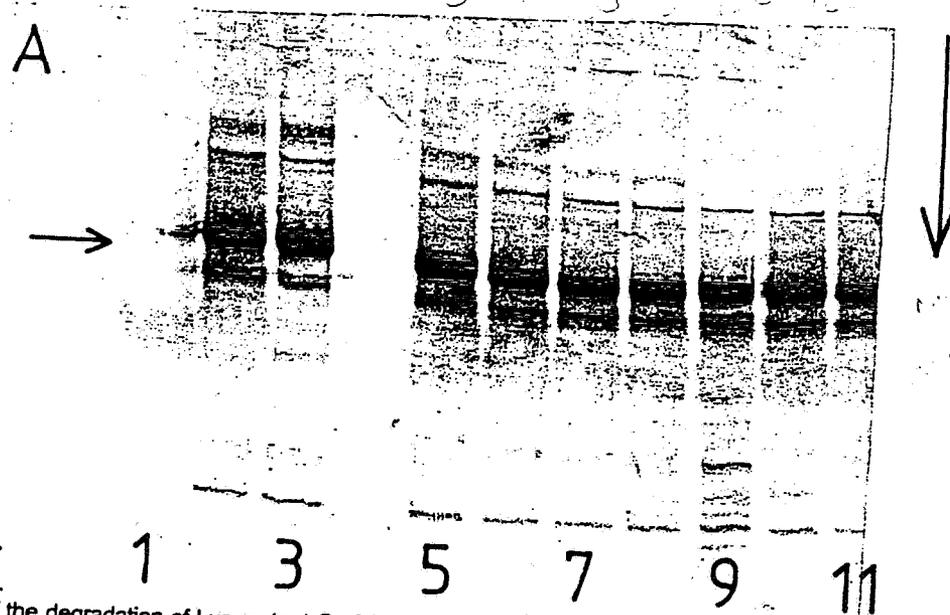


Figure 5. Profile of the degradation of Lys mutant Cry9C protein observed in simulated gastric (panel A) and intestinal (panel B) fluid as analyzed by western blotting (blot, gel nr. 961030 1/2). Figure 5A: lane 1-2, native Cry9C (6.5  $\mu\text{g}/\text{lane}$ ), lane 3, sample of Cry9C (6.5  $\mu\text{g}/\text{lane}$ ) taken at 120 min after incubation in SGF with tomato fruit matrix (10% w/v) at 37°C (pH 2.0), lane 4 quenched simulated gastric fluid with pepsin only, lanes 5-11, samples of Cry9C protein taken at 0 min, at 1 min, at 30 min, at 60 min, at 120 min and at 180 min after incubation at 37°C (pH 2.0) in simulated gastric fluid containing pepsin.

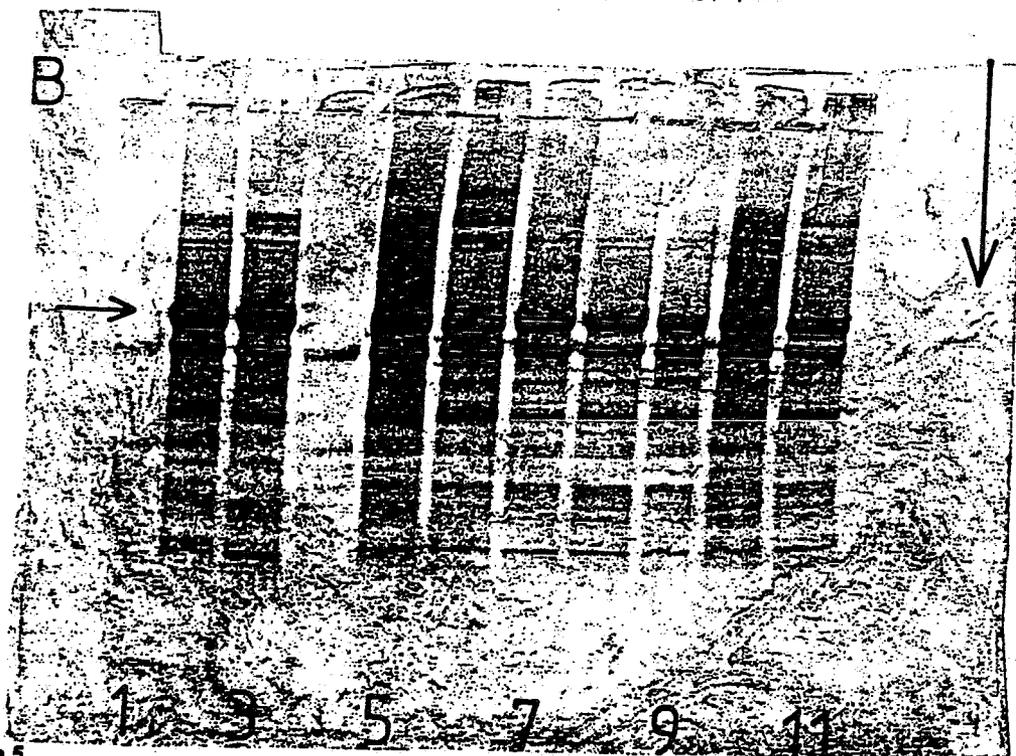


Figure 5. Profile of the degradation of Lys mutant Cry9C protein observed in simulated gastric (panel A) and intestinal (panel B) fluid as analyzed by western blotting (blot, gel nr. 961030 1/2). Figure 5B: lane 1, LMW markers, lane 2, unincubated simulated intestinal fluid with pancreatin and Cry9C, lane 3, sample of Cry9C taken at 120 min after incubation in SIF with tomato fruit matrix (10% w/v) at 37°C (pH 8.0), lane 4, quenched simulated intestinal fluid with pancreatin only, lanes 5-11, samples of Cry9C protein taken at 0 min, at 1 min, at 30 min, at 60 min, at 120 min and at 180 min after incubation at 37°C (pH 2.0) in simulated intestinal fluid containing pancreatin.

FIGURE 6

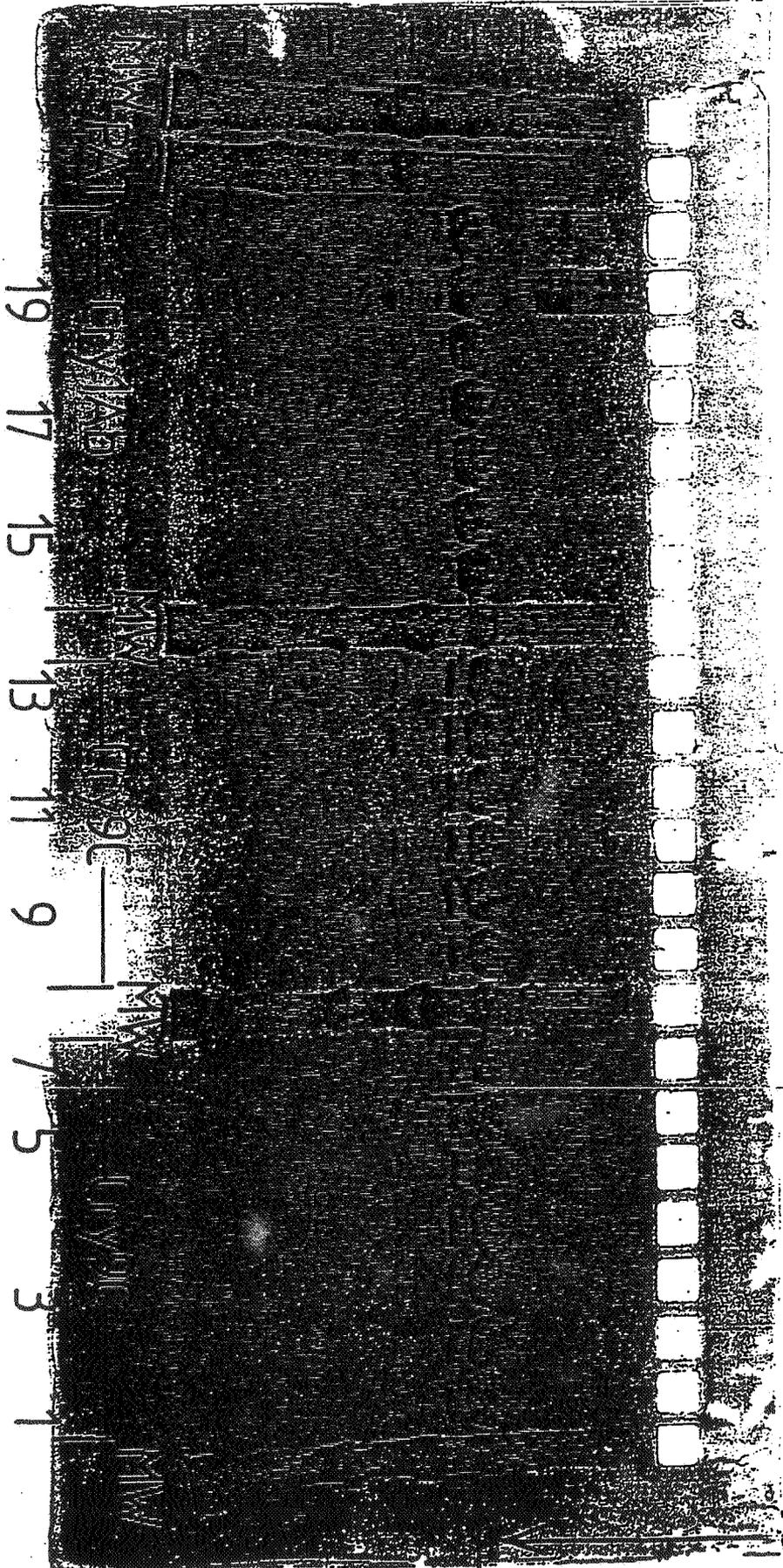


Figure 6. Profile of the thermostability of Lys mutant Cry9C protein observed in simulated processing conditions as analyzed by SDS-PAGE gel electrophoresis (SDS-PAGE, gel nr. 9606252) and stained with silver. Samples: MW, LMW marker proteins (phosphorylase B, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; O-lactalbunin, 14.1 kDa); lanes 1 to 7, Cry9C protein (batch I, 4.4 µg/lane) taken at 0 min (lane 1), at 5 min (lane 2), at 15 min (lane 3), at 30 min (lane 4), at 60 min (lane 5), at 140 min (lane 6) and at 210 min (lane 7) after incubation at 90°C (pH 7.5); lanes 8 to 13, Cry9C protein (batch II, 4.5 µg/lane) taken at 0 min (lane 8), at 5 min (lane 9), at 15 min (lane 10), at 30 min (lane 11), at 60 min (lane 12) and at 210 min (lane 13) after incubation at 90°C (pH 7.5); lanes 14 to 20, Cry1Ab5 protein (3.5 µg/lane) taken at 0 min (lane 14), at 5 min (lane 15), at 15 min (lane 16), at 30 min (lane 17), at 60 min (lane 18), at 140 min (lane 19) and at 210 min (lane 20) after incubation at 90°C (pH 7.5); lane 21, PAT enzyme (4.5 µg/lane) 210 min after incubation at 90°C (pH 7.5).

24 Q

FIGURE 7

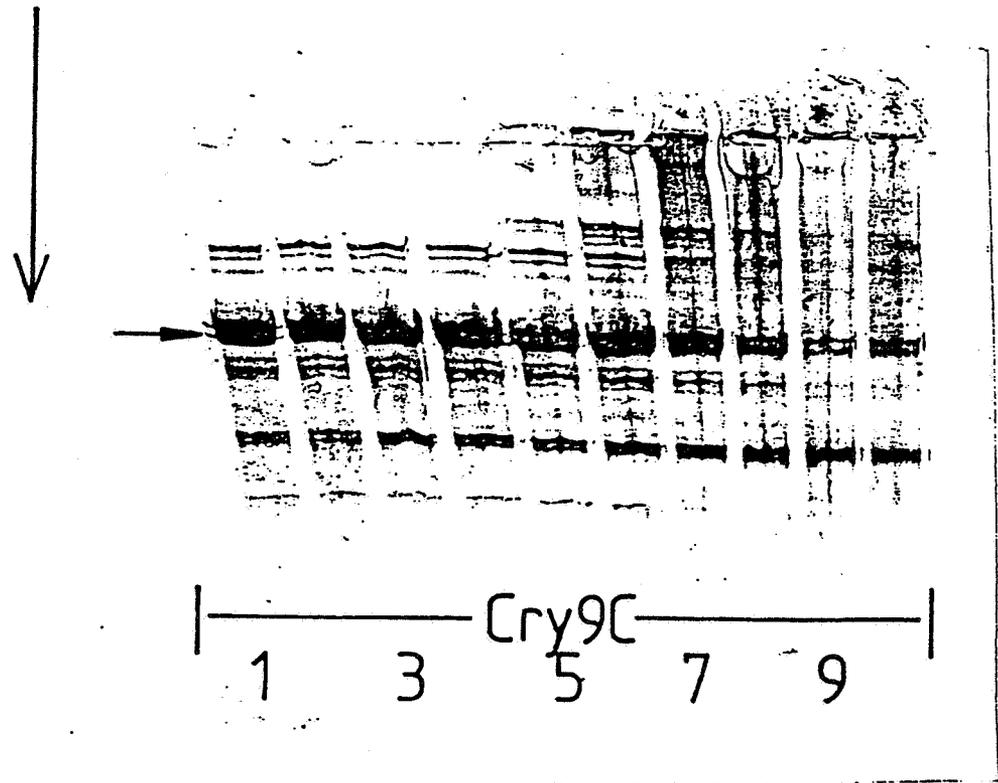


Figure 7.  
Profile of the thermostability of Lys mutant Cry9C protein observed in simulated processing conditions as analyzed by western blotting (blot, gel nr. 960827). Samples: lanes 1 to 3, Cry9C protein (batch I, 4.4  $\mu\text{g}/\text{lane}$ ) taken at 0 min (lane 1), at 60 min (lane 2) and at 210 min (lane 3) after incubation at 20°C (pH 7.5); lanes 4 to 10, Cry9C protein (batch I, 4.4  $\mu\text{g}/\text{lane}$ ) taken at 0 min (lane 4), at 5 min (lane 5), at 15 min (lane 6), at 30 min (lane 7), at 60 min (lane 8), at 120 min (lane 9) and at 210 min (lane 10) after incubation at 90°C (pH 7.5).

24 R  
053

FIGURE 8

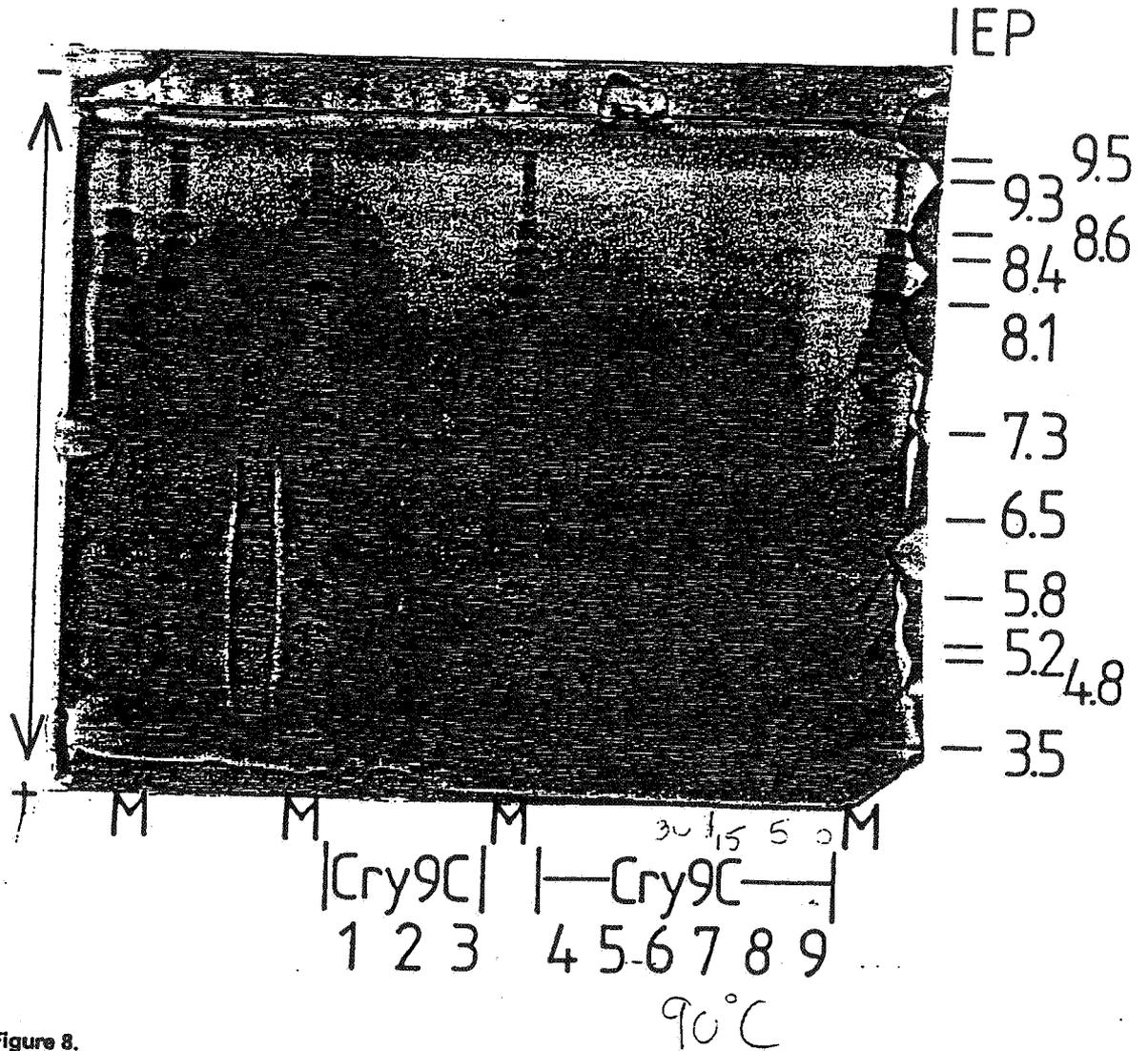


Figure 8. Profile of the thermostability of Lys mutant Cry9C protein observed in simulated processing conditions as analyzed by iso-electric focusing electrophoresis (pH 3.0 - 10.0, gel nr. 960820). Samples: M, IEP markers; lanes 1 to 3, Cry9C protein (batch I, 4.4 µg/lane) taken at 0 min (lane 1), at 60 min (lane 2) and at 120 min (lane 3) after incubation at 20°C (pH 7.5); lanes 9 to 4, Cry9C protein (batch I, 4.4 µg/lane) taken at 0 min (lane 9), at 5 min (lane 8), at 15 min (lane 7), at 30-min (lane 6), at 60 min (lane 5) and at 120 min (lane 4) after incubation at 90°C (pH 7.5).

broader point

FIGURE 9

# BIOAVAILABILITY

## <sup>14</sup>C-PEG4000/<sup>3</sup>H-PROPANOLOL

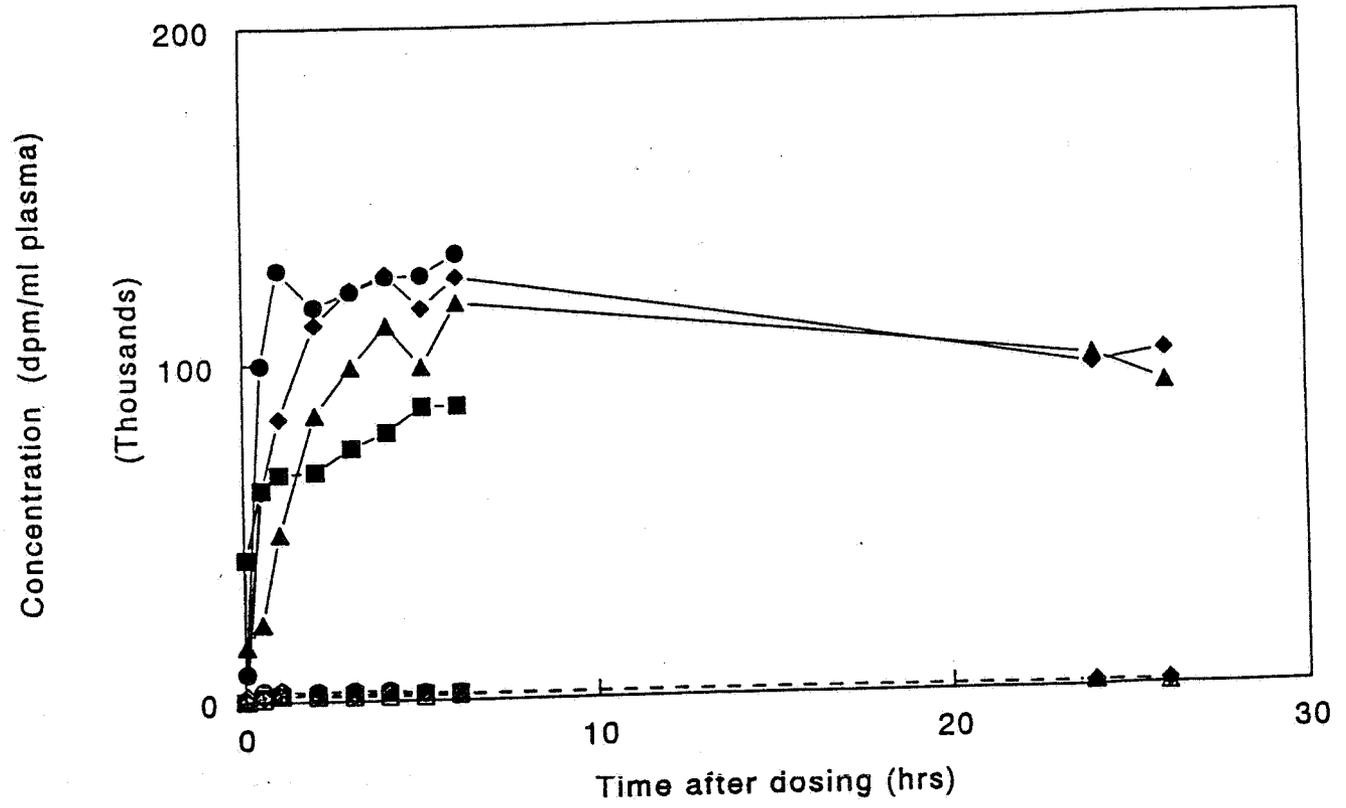
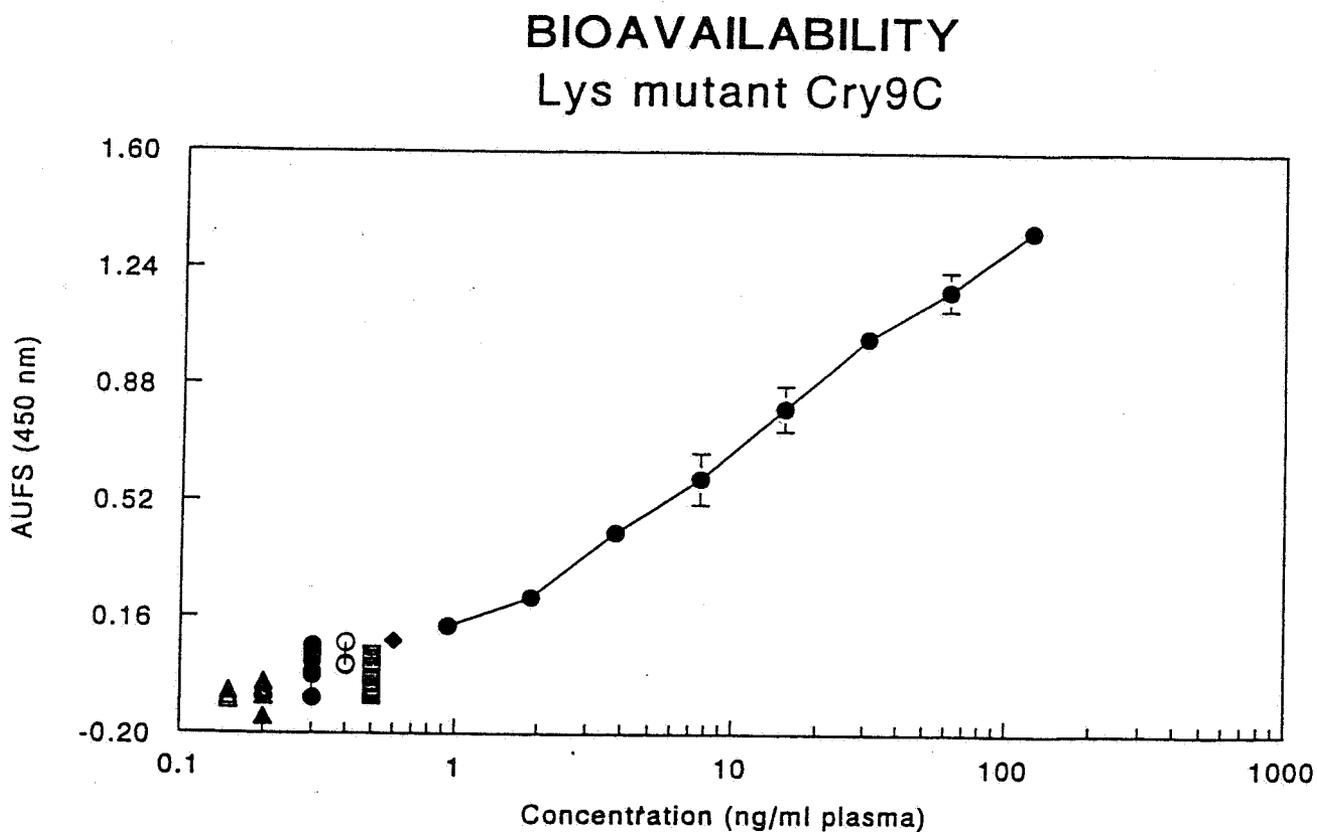


Figure 9. Uptake of [<sup>3</sup>H]propranolol and [<sup>14</sup>C]polyethyleneglycol versus time profiles after oral administration to portal vein cannulated rats. The data represent values from two experiments as described in Annex 1 - Table 3. Key: black symbols values of [<sup>3</sup>H]propranolol (i.e. circles, rat no. 540; squares, rat no. 542; triangles, rat no. 543; diamonds, rat no. 545) and gray symbols values of [<sup>14</sup>C]polyethyleneglycol (i.e. circles, rat no. 540; squares, rat no. 542; triangles, rat no. 543; diamonds, rat no. 545).

24T  
055

HL

FIGURE 10



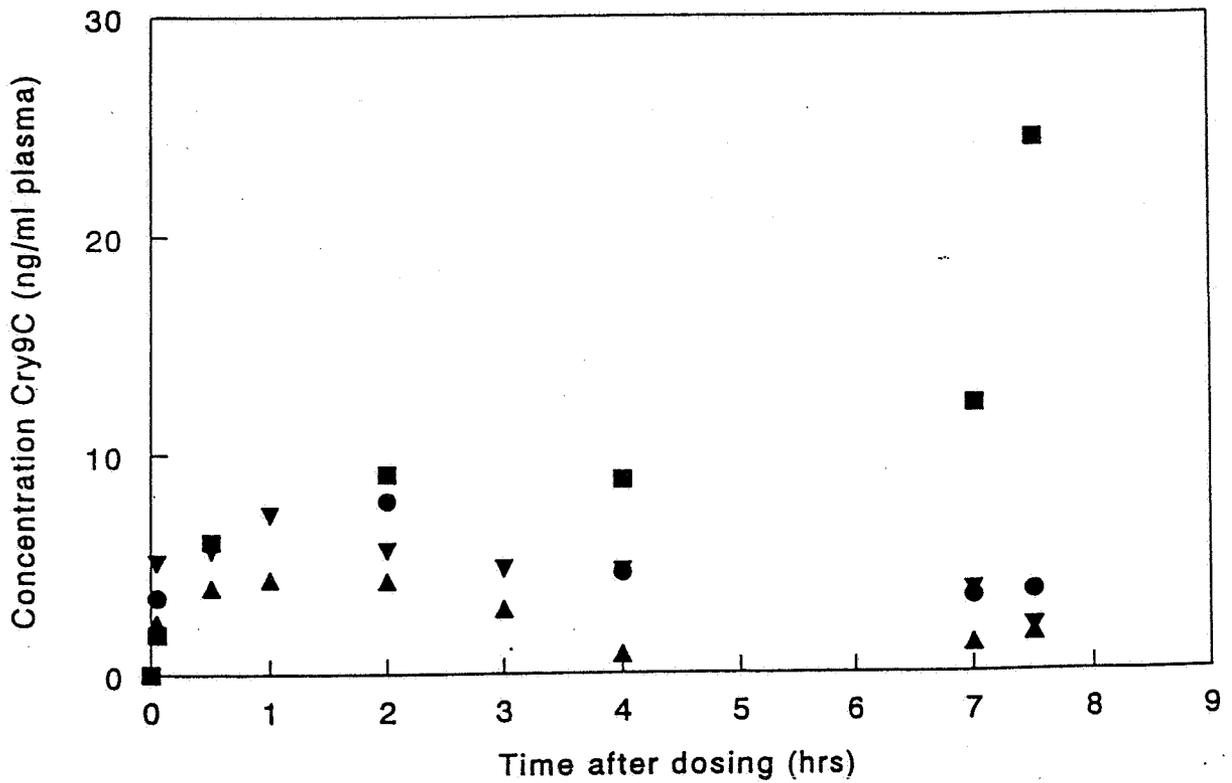
**Figure 10.** Uptake of Lys mutant Cry9C protein versus time profiles after oral administration to portal vein cannulated rats ( $2.6 \pm 0.1$  mg/kg body wt). The data represent values from the experiment as described in Annex 1 - Table 4 (i.e. the individual data points below the level of 1.0 ng/ml of plasma). Key: DS-ELISA standard calibration curve of Lys mutant Cry9C (i.e. black circles); rat no. 521 (open triangles); rat no. 522 (gray squares); rat no. 523 (gray circles); rat no. 524 (gray triangles); rat no. 525 (gray diamonds) and rat no. 528 (open circles). Detection limit: 0.05 AUFS or 0.8 ng/ml of plasma.

056 240

HL

FIGURE 11

## BIOAVAILABILITY Lys mutant Cry9C



**Figure 11.**

Uptake of Lys mutant Cry9C protein versus time profiles after oral administration to portal vein cannulated rats ( $41.9 \pm 1.8$  mg/kg body wt). The data represent values from the experiment as described in Annex 1 - Table 5. Key: rat no. 569 (closed circles); rat no. 573 (closed triangles); rat no. 575 (closed inverted triangles) and rat no. 576 (closed squares). Detection limit: 0.8 ng/ml of plasma.

057 24V

FIGURE 12

# BIOAVAILABILITY

## Lys mutant Cry9C

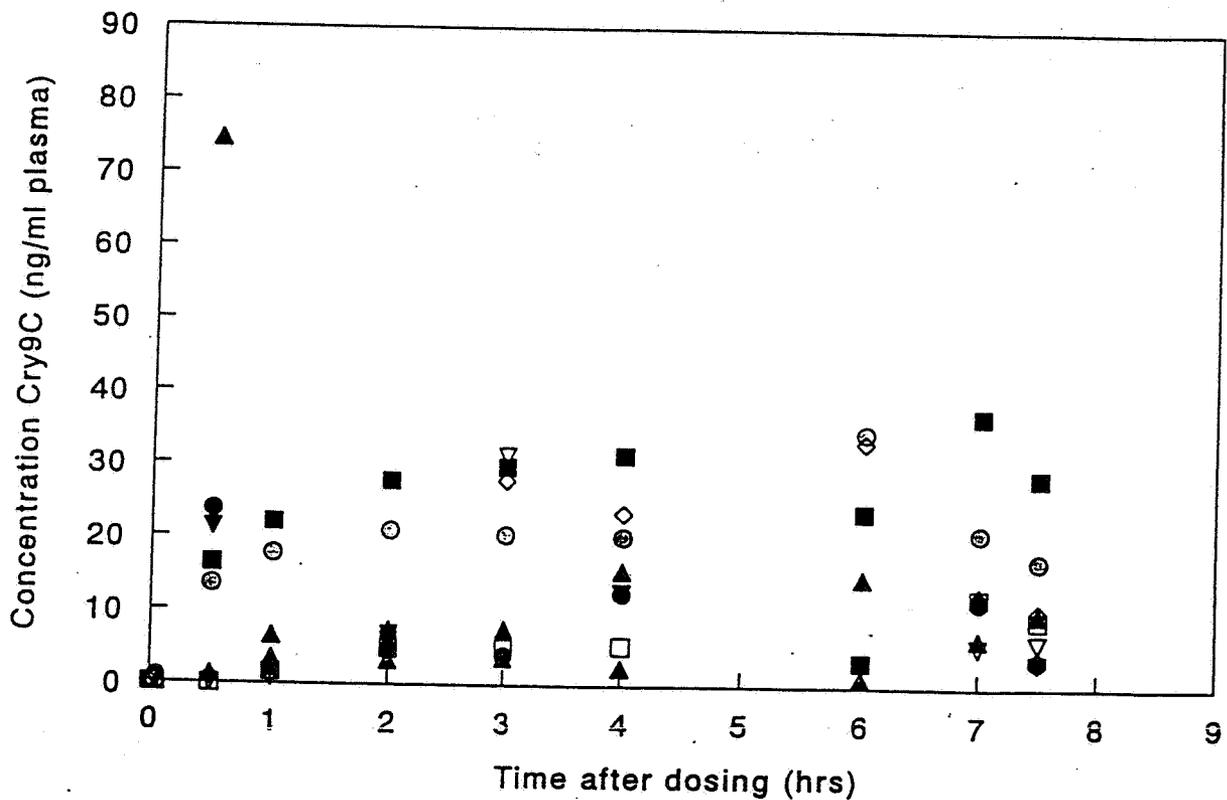


Figure 12.

Uptake of Lys mutant Cry9C protein versus time profiles after oral administration to portal vein cannulated rats ( $272 \pm 27$  mg/kg body wt). The data represent values from the experiment as described in Annex 1 - Table 6. Key: rat no. 547 (closed circles); rat no. 548 (closed triangles); rat no. 549 (closed inverted triangles); rat no. 551 (closed squares); rat no. 556 (gray triangles); rat no. 558 (gray circles); rat no. 561 (open inverted triangles); rat no. 562 (open diamonds) and rat no. 550 (open squares). Detection limit: 0.8 ng/ml of plasma.

058 24w

Figure 13. Digestibility of the LYS mutant Cry9C protein upon G.I.-tract passage in rats as analyzed by western blotting (blot, gel no. 970716). Aliquots of gastric and luminal debris were collected at 8 hours after dosing and separated by SDS-PAGE and the molecular weight and stability of Cry9C was determined by immunoblot analysis. Rat nr. 550, 560, 561 and 551 samples taken from various gut compartments. Key: lanes s, Cry9C in stomic debris, lanes d, Cry9C in duodenal debris, lanes j, Cry9C in jejunal debris, lanes i, Cry9C in ileo debris, lanes c, Cry9C in caecal debris and lanes co, Cry9C in colonic/faecal debris. Lane Cry9C (stock solution, 4.4 ug/lane). The experimental conditions have been described in Annex 1 - Table 6.

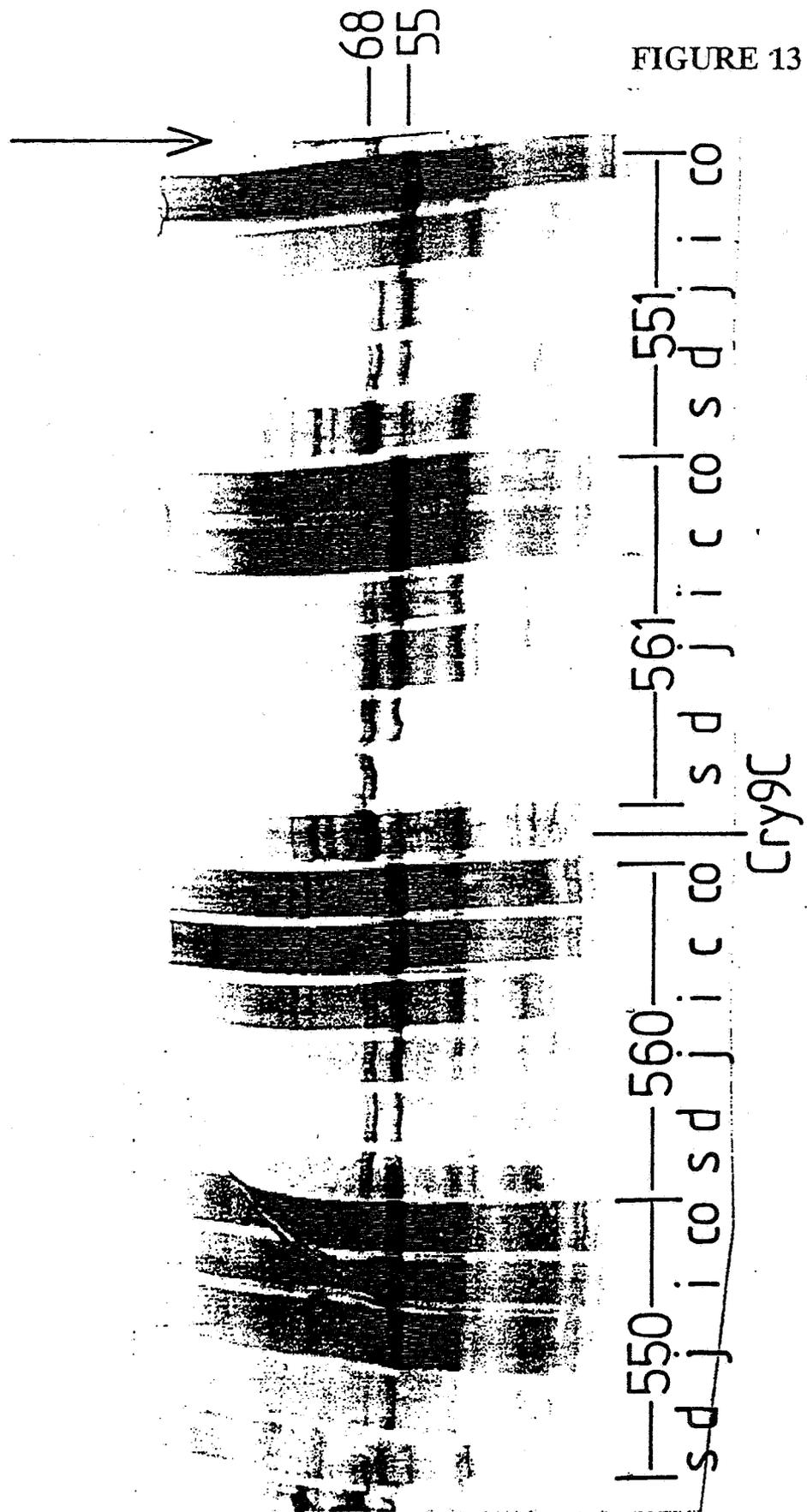


FIGURE 13

059 24\*

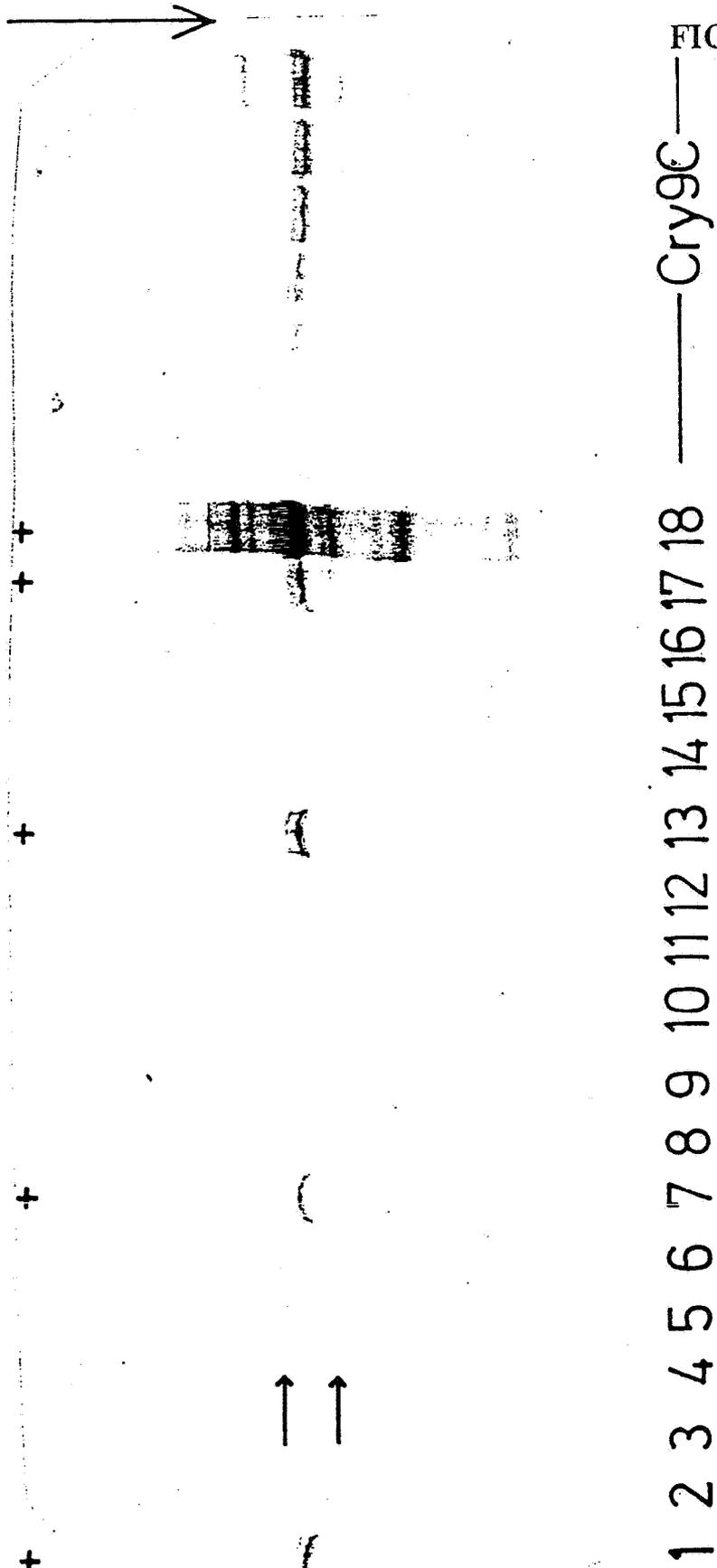


FIGURE 14

Figure 14. Western blot analysis of the LYS mutant Cry9C protein immunoreactivity detected by DS-ELISA in the blood of rats (blot, gel no. 970820A). Aliquots of plasma (about 6  $\mu$ l/lane) collected at various time points after dosing were separated by SDS-PAGE and the molecular weight of the Cry9C-like ELISA immunoreactivities determined by immunoblot analysis. Samples: lane 1 and 13, Cry9C (1.8  $\mu$ g/lane), lane 7, Cry9C (0.9  $\mu$ g/lane) and lane 18, Cry9C (12.0  $\mu$ g/lane); lane 2-4, plasma of rat 551 taken at 0 (lane 2), 7.5 (lane 3) and 7.5 (lane 4) hrs after dosing; lane 5-6, plasma of rat 548 taken at 0 (lane 5) and 0.5 (lane 6) hrs after dosing; lane 8-10, plasma of rat 567 taken at 0 (lane 8), 2 (lane 9) and 7.5 (lane 10) hrs after dosing; lane 11-12, plasma of rat 562 taken at 0 (lane 11) and 6 (lane 12) hrs after dosing; lane 13-16, plasma of rat 558 taken at 0 (lane 13), 3 (lane 14), 6 (lane 15) and 6 (lane 16) hrs after dosing. The experimental conditions have been described in Annex 1 - Table 6.

06024Y

FIGURE 15

## BIOAVAILABILITY Lys mutant Cry9C

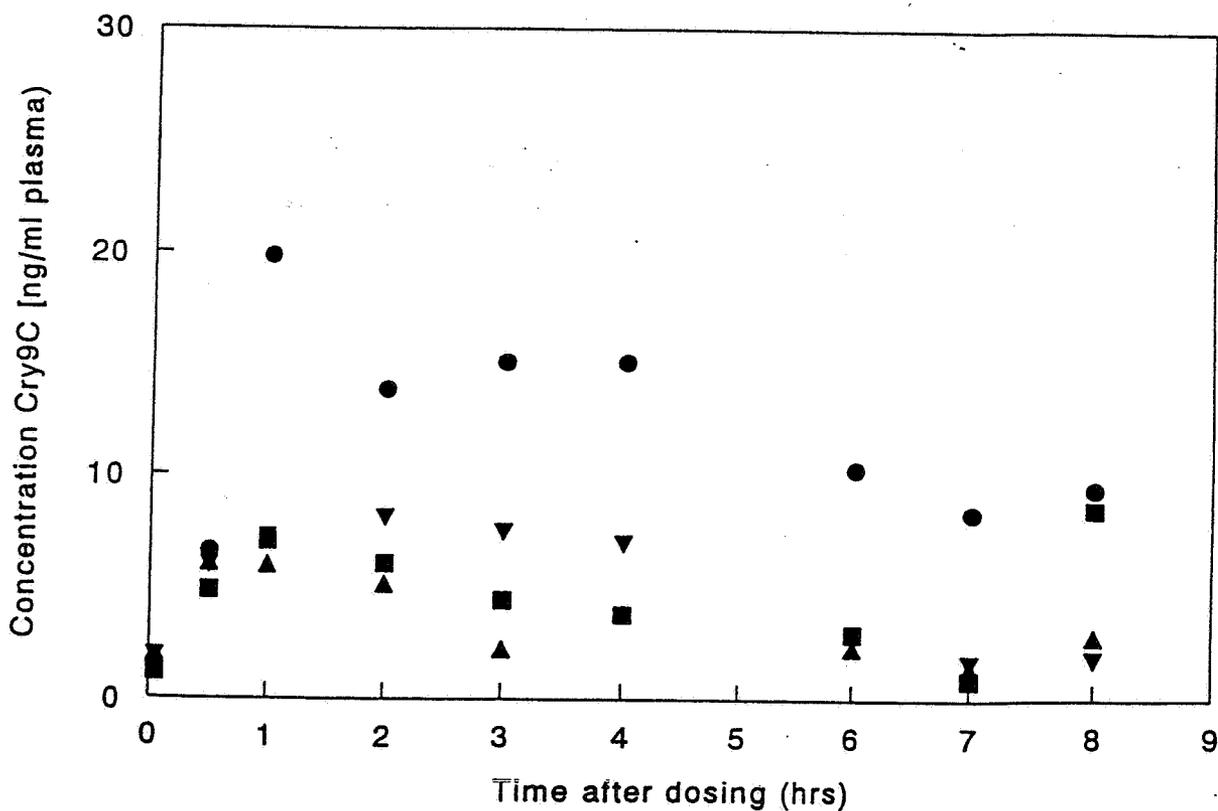


Figure 15.

Uptake of Lys mutant Cry9C protein versus time profiles after oral administration to portal vein cannulated rats ( $298 \pm 6$  mg/kg body wt). The data represent values from the experiment as described in Annex 1 - Table 7. Key: rat no. 565 (closed circles); rat no. 564 (closed triangles); rat no. 574 (closed inverted triangles) and rat no. 568 (closed squares). Detection limit: 0.8 ng/ml of plasma.

061 24Z

FIGURE 16

## BIOAVAILABILITY Lys mutant Cry9C

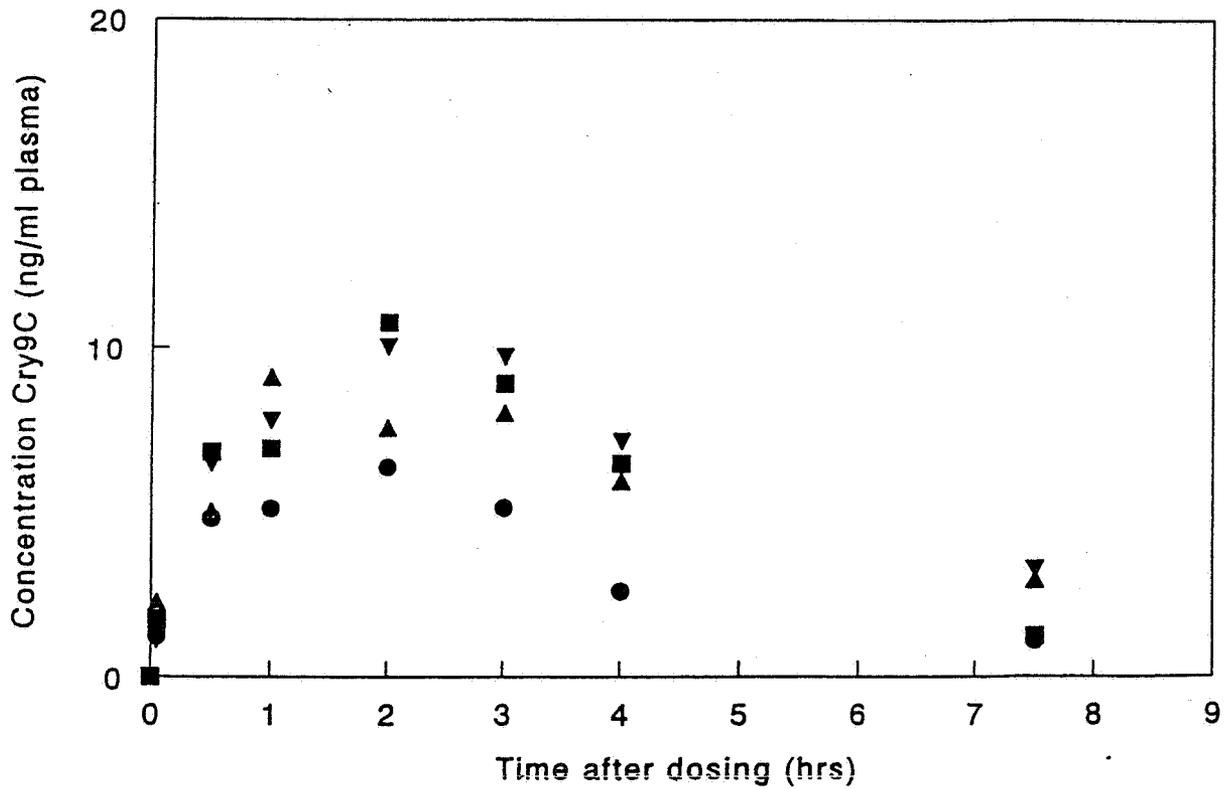


Figure 16.

Uptake of Lys mutant Cry9C protein versus time profiles after oral administration to portal vein cannulated rats ( $198.4 \pm 2.9$  mg/kg body wt). The data represent values from the experiment as described in Annex 1 - Table 8. Key: rat no. 534 (closed circles); rat no. 532 (closed triangles); rat no. 531 (closed inverted triangles) and rat no. 530 (closed squares). Detection limit: 0.8 ng/ml of plasma.

062 24 Aa

HL

FIGURE 17

# BIOAVAILABILITY

## Lys mutant Cry9C

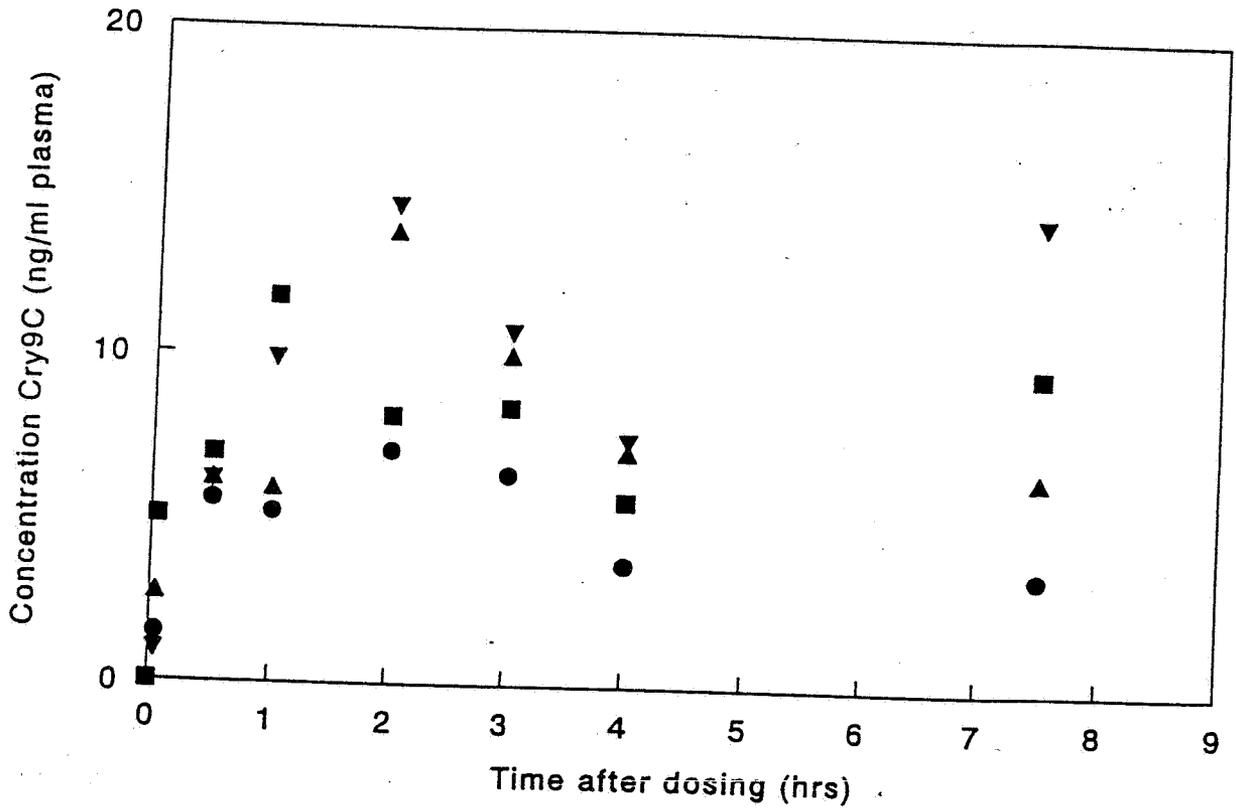


Figure 17.  
Uptake of Lys mutant Cry9C protein versus time profiles after oral administration to portal vein cannulated rats ( $234.7 \pm 8.1$  mg/kg body wt). The data represent values from the experiment as described in Annex 1 - Table 9. Key: rat no. 563 (closed circles); rat no. 577 (closed triangles); rat no. 567 (closed inverted triangles) and rat no. 570 (closed squares). Detection limit: 0.8 ng/ml of plasma.

053 24 Bb

FIGURE 18

# BIOAVAILABILITY

## Lys mutant Cry9C

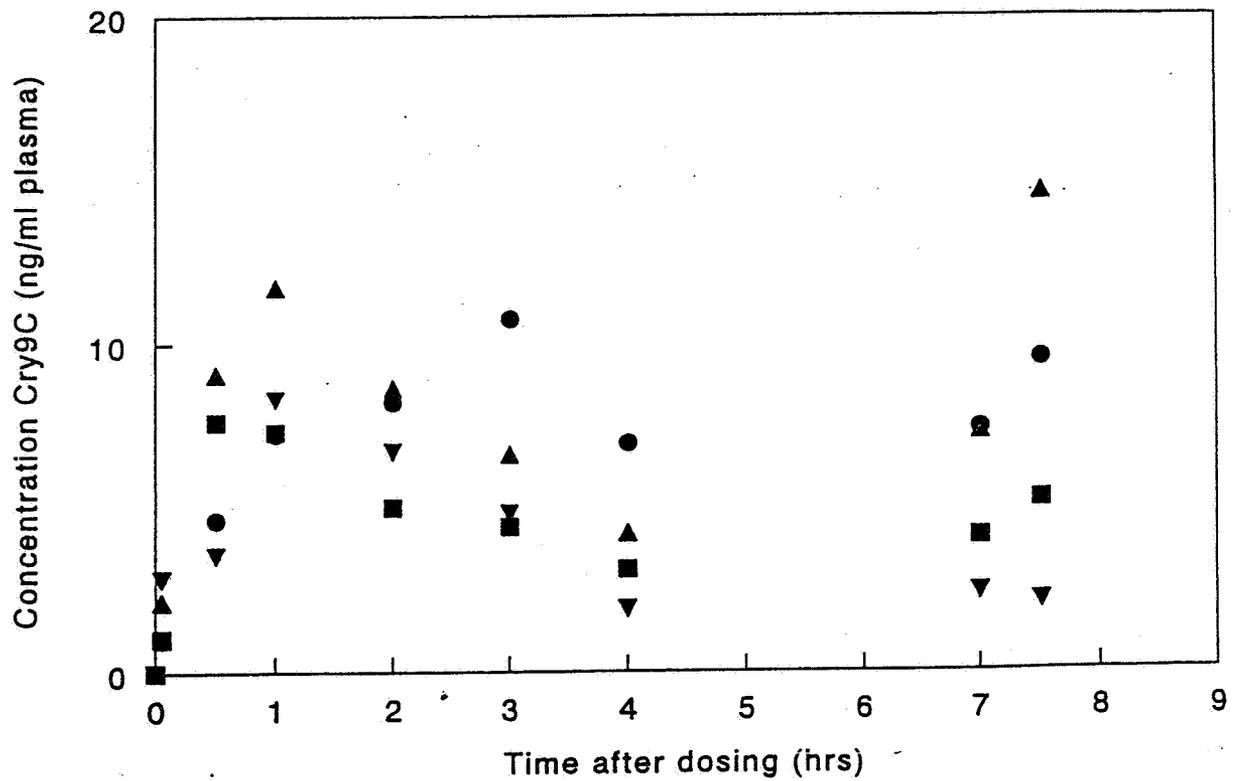


Figure 18.

Uptake of Lys mutant Cry9C protein versus time profiles after oral administration to portal vein cannulated rats ( $229.0 \pm 11.6$  mg/kg body wt). The data represent values from the experiment as described in Annex 1 - Table 10. Key: rat no. 566 (closed circles); rat no. 571 (closed triangles); rat no. 572 (closed inverted triangles) and rat no. 578 (closed squares). Detection limit: 0.8 ng/ml of plasma.

064 24cc

FIGURE 19

### OSMOTIC RESISTANCE OF HUMAN ERYTHROCYTES

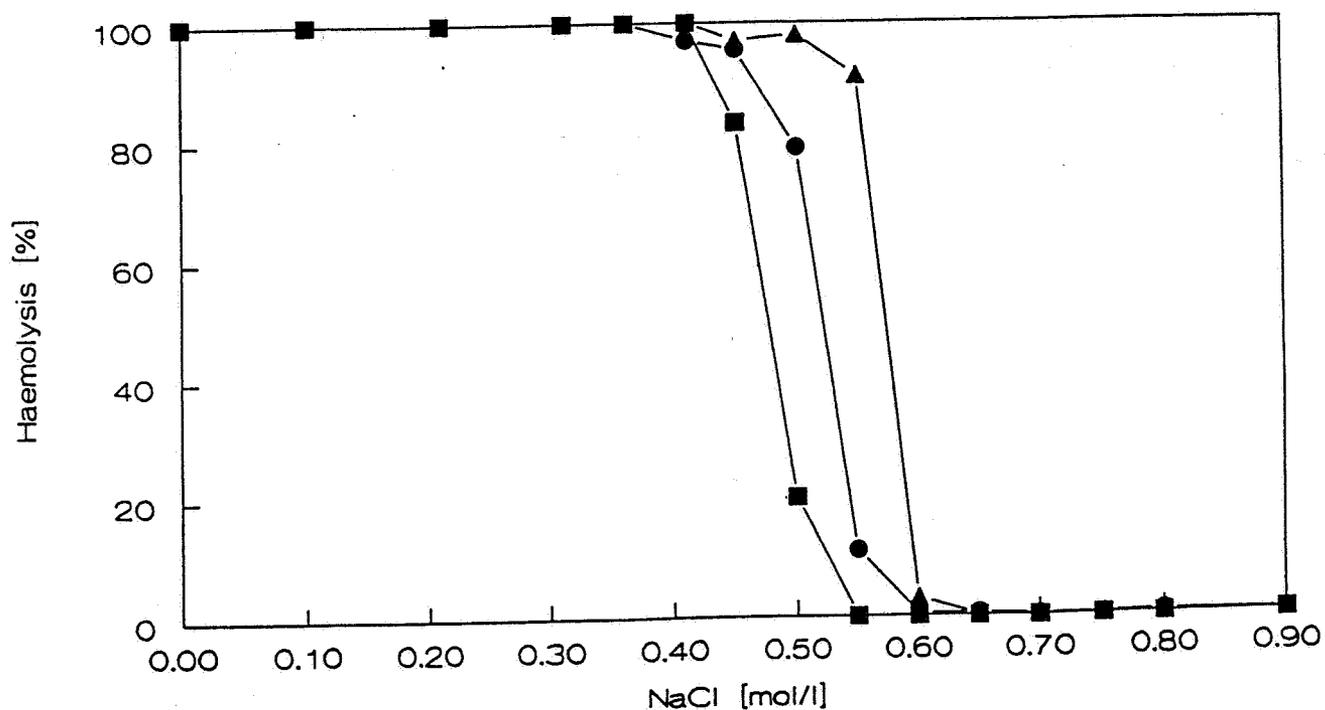


Figure 19. Comparison of haemolysis in different NaCl solutions between human red blood cells in the presence of: (closed squares) 0.5 mg/ml of BSA and (closed triangles) 0.5 mg/ml of Lys mutant Cry9C protein, (closed circles) absence of proteins according to the method of Helleman et al. (1975).

065 24Dd