

**Ethological comparison of the effects of a bovine  $\alpha_{s1}$ -casein tryptic hydrolysate and diazepam on the behaviour of rats in two models of anxiety**

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## **Abstract**

A bovine  $\alpha_{S1}$ -casein tryptic hydrolysate was previously demonstrated to display an anxiolytic-like activity in the conditioned defensive burying and in the elevated plus-maze models when i.p. injected. The present study assessed the anxiolytic-like effects of this tryptic hydrolysate after an oral administration in rats faced to the same behavioural situations using diazepam as a reference. In a first experiment, the behavioural effects of the hydrolysate in the conditioned defensive burying test were investigated at doses ranging 5-50 mg/kg. The results showed that the minimal dose required to elicit an anxiolytic-like activity is 15 mg/kg. In a second experiment, the  $\alpha_{S1}$ -casein tryptic hydrolysate (15 mg/kg, p.o.) was demonstrated to display an anxiolytic-like activity similar to diazepam (3 mg/kg, p.o.) in the conditioned defensive burying test and the elevated plus-maze. However, the ethological analysis of behaviour indicated that this hydrolysate has a different activity compared to diazepam. While diazepam induced a disinhibition state in rats, possibly related to the risk-taking behaviour observed after a benzodiazepine ingestion in humans, the tryptic hydrolysate did not display such a side effect. These results suggest that the mechanism of action of the bovine  $\alpha_{S1}$ -casein tryptic hydrolysate may differ from that of diazepam.

*Keywords:* Bovine  $\alpha_{S1}$ -casein tryptic hydrolysate; Diazepam; Anxiety; Conditioned defensive burying; Elevated plus-maze; Rat; Risk-taking behaviour.

## 1. Introduction

Milk proteins are the only proteins synthesised by mammals in order to feed their offspring. Beyond their nutritional importance, these proteins, and particularly caseins, are now largely recognised as a source of bioactive peptides that have been shown to play physiological roles in both peripheral and central systems. These bioactive peptides can be released during digestion or manufactured *in vitro* by specific enzyme-mediated proteolysis (Pihlanto-Leppala, 2001; Silva and Malcata, 2005). They can act as mineral carriers (Meisel and FitzGerald, 2003; Sato et al., 1986), opioids (Chiba and Yoshikawa, 1986; Meisel and FitzGerald, 2000; Teshemacher, 2003; Zioudrou et al., 1979), immunoregulators (Gill et al., 2000), but also as antimicrobial, anti-thrombotic or anti-hypertensive agents (Clare et al., 2003; Rutherford and Gill, 2000; Sipola et al., 2002; Takano, 2002; Zucht et al., 1995).

Recently, a bovine  $\alpha_{S1}$ -casein tryptic hydrolysate (CTH) was demonstrated to have an anxiolytic-like activity in the conditioned defensive burying (CDB) test and the elevated plus-maze (EPM) when *i.p.* injected to the rats (Miclo et al., 2001). Results obtained from both pre-clinical and clinical studies also suggest the ability of the CTH to protect individuals from the effects of different stressful situations (Guesdon et al., *in press*; Messaoudi et al., 2005).

This CTH is supposed to act through the  $\gamma$ -amino butyric acid type A (GABA<sub>A</sub>) receptor for the following reasons: firstly, it protects rats from the pentylenetetrazole-induced seizures; secondly, the CTH contains a peptide corresponding to the  $\alpha_{S1}$ -casein f(91-100), that has been demonstrated to bind to the benzodiazepine site of the GABA<sub>A</sub> receptor (Miclo et al., 2001); and thirdly, this peptide shows some conformational similarities with nitrazepam (Lecouvey et al., 1997).

However, the question of the anxiolytic-like activity of the CTH and the  $\alpha_{S1}$ -casein f91-100 after an oral administration remains to be elicited. Indeed, it is classically recognised that only di- or tripeptides can cross through the intestinal barrier (Pihlanto-Leppala, 2001). Nevertheless, longer peptides as the  $\alpha_{S1}$ -casein f1-23 have been demonstrated to be present in blood after milk ingestion and to resist to plasmatic proteases in humans (Chabance et al., 1998). More recently, it has been demonstrated that enkephalin-related peptides composed of 6 to 15 amino acids are able to cross through the blood brain barrier and act on the central nervous system (Egleton et al., 2005). Thus, the persistence of the anxiolytic-like activity of the CTH after an oral administration would indirectly confirm that the  $\alpha_{S1}$ -casein f91-100 may cross through the biological barriers and modify the central nervous system activity.

The present study was designed to confirm the anxiolytic-like effect of this hydrolysate when orally administered in two very useful behavioural models that are relevant for the investigation of anxiety in rat, the CDB test (Craft et al., 1988; Treit, 1985; Treit et al., 1981) and the EPM (Pellow et al., 1985), and to compare it with the behavioural effects of the well-known anxiolytic diazepam (DZ).

For this purpose, two experiments were conducted. In the first one, the dose response characteristics of the CTH oral administration on anxiety-related behaviour was evaluated in the CDB test in order to determine the minimal anxiolytic dose. In the second experiment, the behavioural effects of CTH (15mg/kg p.o.) and DZ (3 mg/kg p.o.) were investigated in the CDB test and the EPM.

In these two tests, we used an ethological approach consisting in scoring specific rat behaviours in addition to the classical observed parameters like the probe-burying duration or the time spent in the open arms. This approach allows a more accurate characterisation of the pharmacological properties of psychoactive drugs (Cruz et al.,

1994; De Boer and Koolhass, 2003; Rodgers and Johnson, 1995; Rodgers et al., 1997a, 1997b).

Besides, the combination of these two behavioural paradigms is interesting in permitting the exploration of different aspects of anxiety: the burying duration that is scored in the CDB test corresponds to an anxiety-induced active avoidance (burying a potentially dangerous element), whereas the time spent in the open arms, the classical variable scored in the EPM, rather corresponds to an anxiety-induced passive avoidance (avoiding a non-protected area by staying in the closed arms). Consequently, the administration of an anxiolytic drug leads to the expression of opposite behaviours in these two situations: in the CDB test, a decrease of anxiety is related to a behavioural inhibition (the rat stops to bury the probe), whereas in the EPM, a decrease of anxiety corresponds to a behavioural disinhibition (the rat exits more often in the open arms).

Thus, these tests take advantage of different aspects of animal behaviour while having a distinct complement of problems associated with interpretation (Dawson and Trickelbank, 1995; Wilson et al., 2004).

## **2. Material and methods**

### *2.1. Animals*

One hundred Wistar male rats weighing 280-300 g (AF/EOPS, Charles River Laboratories, L'Arbresle, France) were used for the present study. They were housed by pairs in a regulated environment ( $20 \pm 1^\circ\text{C}$ ; humidity  $50 \pm 5\%$ ; light on from 8:00 p.m. to 8:00 a.m.) and subjected to a seven-day acclimatisation period before study. Food pellets (Dietex, Saint Gratien, France) and tap water were provided ad libitum. All rats were handled in the same way, under the same conditions, and were randomly allocated

to treatment groups. All procedures were in compliance with the rules provided by the European Communities Council Directive of 24 November 1986 (86/609/EEC).

## *2.2. Drugs*

The CTH (LACTIUM<sup>TM</sup>, Ingredia, Arras, France) was obtained through a food grade industrial process adapted from Miclo et al. (1998). The CTH was delivered as a free flowing, pale cream powder, containing 5.5% of water, less than 1% of fat, 75% of proteins and 15% of minerals. DZ was purchased from Roche (VALIUM<sup>®</sup>, Neuilly-sur-Seine, France). CTH (5, 15, 30 and 50 mg/kg) and DZ (3 mg/kg) were suspended in a 0.5% methylcellulose aqueous solution and were orally administered in a volume of 5 ml/kg body weight. Control rats received 5 ml/kg of a methylcellulose solution under the same conditions.

## *2.3. Conditioned defensive burying test*

### *2.3.1. Test design*

The testing was done in a 44 x 28 x 18 cm (L x l x h) clear Plexiglas chamber under a red dim light. The floor of the cage was evenly covered with 5 cm of bedding material made of wood sawdust. On the centre of one wall, 2 cm above the level of the bedding material, a small hole allowed to insert a shock-probe. The shock-probe (7 x 2 x 0.5 cm, L x l x h) overlaid with a copper wire-integrated circuit was connected to a two-pole shock generator (Intellibio, Nancy, France). Rats were familiarised with the apparatus by placing each pair of rats in the test chamber without the shock-probe for 20 min per day, the previous two days before testing. The next day, each rat was tested alone. For that, the shock-probe was inserted into the test chamber before the test session and the rat was placed in the chamber facing away from the shock-probe. Then,

the experimenter delivered a single 2-mA aversive electric shock when the animal touched it with its forepaws for the first time. Immediately after, the behaviour of the rat was recorded for 5 min.

### *2.3.2. Behavioural scoring*

Behaviours were scored from videotapes by a trained experimenter who was unaware of the administered treatment. Three variables were quantified in this paradigm: the time duration of probe-burying (the rat pushes the sawdust with its forelimbs in the direction of the probe), the latency of the first probe approach after the shock, and the latency of the first probe contact after the shock.

## *2.4. Elevated plus-maze*

### *2.4.1. Test design*

The apparatus was made of Plexiglas. It consisted of a maze elevated to the height of 70 cm with two open (50 x 10 cm) and two closed arms having walls (50 x 10 x 50 cm), arranged so that arms of the same type were opposite to each other. The four arms extended from a common central platform (10 x 10 cm). To prevent rats from falling out of the maze, a ledge of Plexiglas (2 mm of height) surrounded the open arms. For the test, the rat was placed at the centre of the apparatus, head turned towards an open arm, then it was let freely walking for 5 min. All the tests were conducted under red dim light, and recorded by video camera. Videotapes were subsequently scored by a trained observer using an ethological analysis software developed in the laboratory. Real time scores of videotapes were made by direct keyboard entry to a computer.

#### *2.4.2. Behavioural scoring*

Standard spatiotemporal measures were scored: the number of entries in the open and the closed arms, the total number of arm entries and the time spent in the different parts of the maze (open and closed arms). The ratio of open arm entries and closed arm entries to the total number of arm entries was also calculated.

In addition, some more ethologically orientated measures were observed according to Cruz et al. (1994) and Rodgers and Johnson (1995): the number of head dips (the rat stretches his head over the ledge of an open arm and bend it under the maze floor) and the number of rears (the rat raises its body on the hind limbs in a vertical position). Also, the number of head dips, the number of rears and the rearing duration were scored in the different parts of the maze, and their frequencies of appearance depending on their localisation were calculated.

#### *2.5. Experimental procedure*

##### *2.5.1. Experiment 1 : Dose response of bovine $\alpha_{S1}$ -casein tryptic hydrolysate using the conditioned defensive burying test*

Forty rats were used for this experiment, and were divided into five groups of 8 animals each: control, CTH 5, 15, 30 and 50 mg/kg. The rats were orally treated 1 h before testing. The CDB test was performed during the first three hours of the dark period.

##### *2.5.2. Experiment 2 : Behavioural effects of bovine $\alpha_{S1}$ -casein tryptic hydrolysate and diazepam in the conditioned defensive burying test and the elevated plus-maze*

Sixty rats were used for this part of the present study: 30 for the CDB experiment, and 30 for the EPM testing. For each behavioural testing, 3 groups of 10 rats were constituted: control, DZ and CTH. Based on the results of the first experiment, the dose

of 15 mg/kg was chosen for the hydrolysate, whereas DZ was administered at the anxiolytic dose of 3 mg/kg (Chen et al., 2005; Gries et al., 2005). The animals were orally treated 1 h before testing. The two tests were performed during the first three hours of the dark period.

## 2.6. Statistical analysis

Results are expressed as mean  $\pm$  SEM. For each variable, the effects of treatments were analysed using a one-way analysis of variance (one-way ANOVA) followed by a Scheffé's test for multiple comparisons. The statistical analysis was performed using the SPSS 11.5 for Windows software (SPSS inc., Chicago, Illinois, USA). Differences were considered to be significant at the level of  $p < 0.05$ .

## 3. Results

### 3.1. Experiment 1: Dose response of bovine $\alpha_{S1}$ -casein tryptic hydrolysate using the conditioned defensive burying test

The one-way ANOVA showed a significant difference in the probe burying duration between the five groups,  $F(4, 35) = 15.25, p < 0.01$ . As indicated in Fig. 1A, rats treated with CTH at the doses of 15, 30 and 50 mg/kg displayed lower probe burying durations than controls, whereas the animals receiving 5 mg/kg of CTH did not. In the same way, a significant difference in the latency of the first probe approach after the shock was observed between the five groups,  $F(4, 35) = 7.39, p < 0.01$ . Post-hoc comparisons showed that this latency was significantly lower only in groups treated with 15, 30 and 50 mg/kg compared to controls (Fig. 1B).

Concerning the latency of the first probe contact after the shock, the statistical analysis failed to show a significant group effect (Fig. 1C).

### *3.2. Experiment 2: Behavioural effects of bovine $\alpha_{S1}$ -casein tryptic hydrolysate and diazepam in the conditioned defensive burying test and the elevated plus-maze*

#### *3.2.1. Conditioned defensive burying test*

The statistical analysis showed a significant difference in the probe burying duration between the three groups,  $F(2, 29) = 6.84, p < 0.01$ . As indicated in Fig. 2A, both rats treated with CTH (15 mg/kg, p.o.) and DZ (3 mg/kg, p.o.) displayed lower probe burying durations than control animals.

In the same way, a significant difference in the latency of the first probe approach after the shock was observed between the three groups,  $F(2, 29) = 7.89, p < 0.01$ . Post-hoc comparisons showed that this latency was significantly lower in CTH- and DZ-treated rats compared to controls (Fig. 2B). Concerning the latency of the first probe contact after the shock, the one-way ANOVA also showed a significant variation between the three groups,  $F(2, 29) = 6.83, p < 0.01$ . However, the Scheffé's post-hoc tests indicated that DZ induced a significant ten-fold decrease of this delay compared to controls, whereas the CTH did not (Fig. 2C).

#### *3.2.2. Elevated plus-maze*

As indicated in the Table 1, the one-way ANOVA showed a significant effect of the treatments on different behavioural variables scored in this maze as the total number of arm entries, the percentage of open arm entries, the percentage of closed arm entries, the percentage of the time spent in the open arms, the total number of head dips, the number of head dips performed in the open arms, the number of rears and the rearing duration in the open arms.

In both DZ- and CTH-treated rats, significant increases were observed in the percentage of open arm entries (+85% and +117% respectively), and in the percentage of the time spent in the open part of the maze (+107% and +86%) compared to controls. On the opposite, the percentage of closed arm entries was significantly decreased (-36% and -49%, respectively) (Table 1).

Scheffé's test also showed that DZ induced significant increases compared to controls in the total number of arms entries (+59%), the total number of head dips (+118%), the number of head dips and the number of rears performed in the open arms (+172% and +229% respectively), and the rearing duration in this part of the maze (+374%), whereas the administration of the CTH did not (Table 1). It also should be noticed that the total number of arms entries was significantly increased in the DZ-treated rats compared to the CTH-administered animals (+61%) (Table 1).

#### **4. Discussion**

In the present study, the anxiolytic-like effect of the oral administration of a CTH was investigated in the CDB test and in the EPM. In these two models, the CTH administered at the dose of 15 mg/kg showed anxiolytic-like properties but with a different behavioural profile compared to DZ. This dose of CTH was chosen because of the results we obtained in Experiment 1. Indeed, 15 mg/kg appeared to be the minimal dose able to significantly reduce the anxiety level of rats in the CDB.

As previously described, the well-known anxiolytic DZ inhibits the probe burying behaviour of rats subjected to the CDB task (Treit et al., 1981; Wilson et al., 2004). In the EPM, DZ shows an anxiolytic profile as reflected by the increases of both the time the rats spent in the open arms and the number of open arm entries they did (Cosquer et al., 2005; Wilson et al., 2004). Regarding these behavioural items, the CTH

induces the same behavioural changes in rats suggesting a potent anxiolytic-like activity that is very close to DZ.

The combination of the EPM and the CDB test decreases the risk of a 'false positive' result: in the present study, the CTH anxiolytic-like effect observed in the EPM is confirmed by the results measured in the CDB task. Indeed, the EPM has been demonstrated to be sensitive to various psychoactive substances such as anxiolytics (Rodgers et al., 1997a, 1997b), psychostimulants (Dawson and Trinckelbank, 1995; El Yacoubi et al., 2000) or anesthetics (Kurt et al., 2003) whereas the CDB has not (De Boer and Koolhass, 2003). Moreover, the results observed in both situations rule out the ambiguity between a possible analgesic activity and an anxiolytic-like effect of the CTH in the CDB test which is a paradigm based on a potentially painful electrical shock (Craft et al., 1988).

The ethological investigation of the rat behaviour also confirms this conclusion. In the EPM, DZ and the CTH increase the number of head dips in the open arms, a specific behaviour that was demonstrated to correlate negatively with the anxiety level of rats (Cruz et al., 1994; Rodgers and Jonhson, 1995). In the same way, the latency of the first approach towards the probe, an item that is considered to reflect anxiety in the CDB test (De Boer and Koolhass, 2003), is decreased by both treatments.

These results are in good accordance with those obtained by Miclo et al. (2001) who demonstrated an anxiolytic activity for the CTH in these two behavioural situations at a dose of 3 mg/kg i.p. In the present study, we showed that the CTH is also active after an oral administration at a dose of 15 mg/kg. This confirms the efficiency of the CTH when using this way of administration, as suggested by the results obtained in both rats and humans exposed to a stressfull situation (Guesdon et al., in press; Messaoudi et al., 2005).

However, the mechanisms underlying the anxiolytic activity of the CTH remain unknown. An attractive hypothesis is that one or more active compounds may be encrypted in the CTH fragments and be released at the peripheral level. Currently, a peptide corresponding to the 91-100 fragment of the bovine  $\alpha_{S1}$ -casein is supposed to significantly contribute to the biological activity of the CTH. As described in the introduction, this peptide has been demonstrated to bind to the benzodiazepine site of the GABA<sub>A</sub> receptor and to display an anxiolytic activity in CDB test when i.p. injected (Miclo et al., 2001). Moreover, Lecouvey et al. (1997) has demonstrated that the conformation of the four amino-acids of the N-terminal domain of this peptide presents some similarities with nitrazepam, suggesting that the C-terminal domain may be cleaved without a loss of the pharmacological activity. Thus it may be hypothesised from the present study that this complete peptide, or at least a part of it issued from a peripheral hydrolysis, may cross through the biological barriers and act on the central nervous system. Recently, Chabance et al. (1998) detected two peptides longer than 10 amino acids in blood several hours after milk or yoghurt ingestion, suggesting that these peptides can cross into the blood despite a high number of amino-acids, and may resist to plasmatic proteases. Several studies has also demonstrated in vitro and in vivo that peptides longer than 10 amino-acids with amphipathic properties can cross through the blood brain barrier, and then modify the central nervous system activity (Egleton et al., 2005; Sikiric et al., 2001).

In addition, the behavioural effect of the CTH was not the same as DZ. In the EPM, the total number of arm entries, generally considered as reflecting the locomotor activity of the animals (Cruz et al. 1994; Rodgers and Johnson, 1995), was significantly increased after the administration of DZ whereas it remained unchanged with the CTH. The DZ stimulatory action on the locomotor activity in the EPM was also reported in

rodents as rat (Silvestre et al., 1999), mouse (Belzung and Agmo, 1997) and gerbil (Varty et al., 2002). At least, these results suggest that CTH pharmacological profile is different from DZ.

Such difference is also observed in the CDB test. In this task, the latency of the first contact with the probe after the shock was highly reduced in DZ-treated rats but not in CTH-treated animals, whereas the latency of the first approach was similarly reduced within the two groups. These results suggest that DZ, but not the CTH, induces a disinhibition state in rats that may lead them to express non-adaptive behaviours. After the shock, the probe can be considered as an aversive object for the animals, leading them to avoid it. With DZ, the animals approached the probe (mean latency = 3.4s) and immediately touched it (mean latency = 15s). On the contrary, the CTH-treated rats performed some approaches (mean latency = 6.7s) but avoid any early contact with the probe as reflected by a higher latency (mean = 110s). This hypothesis is also confirmed by the results of the first experiment which showed that the animals expressed the same behaviour despite the increase of the dose administered. The results we observed with DZ are in accordance with numerous epidemiological and clinical studies in humans that demonstrated the implication of the benzodiazepine in the emergence of risk-taking behaviours, including aggression (Bond, 1998), violent injuries, often in a social setting (MacDonalds et al., 1999), motor vehicle accident (Van Laar and Volkerts, 1998), sexual assault (Calhoun et al., 1996) and HIV-risk behaviours in methadone maintenance patients (Bleich et al., 1999). More recently, some experimental studies using standardised risk-taking tasks (games of chance based on money wagering), showed that the acute administration of benzodiazepine produces an increase in risk-taking behaviours in healthy humans (Deakin et al. 2004; Lane et al., 2005). The results obtained from the EPM are able to confirm this hypothesis. Indeed, the DZ

administration induced some increases in behaviours related to the vertical exploration of the maze in the open arms (rears and head dips), with a higher risk of falling out of the maze. Thus, all these results suggest that the CTH do not induce in rats a disinhibition state as observed with DZ.

In conclusion, the results of the present study suggest that the oral administration of a CTH is acting as an efficacious anxiolytic compound close to DZ in the EPM and the CDB test. However, the pharmacological activity of the CTH in these two situations was different from DZ. In particular, DZ induced a high disinhibition state in rats, whereas the CTH did not. The contrast observed between the action of this two agents suggests that the mechanisms underlying their respective central activities are different. Although the CTH activity is supposed to be supported by the 91-100 benzodiazepine-like peptide it contains, the specific modulation of the GABAergic system induced by this ligand remains to be investigated.

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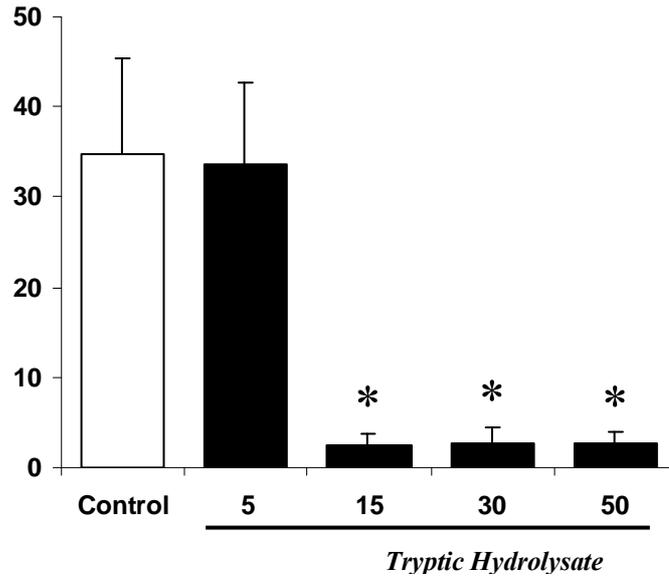
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## Legend of figures

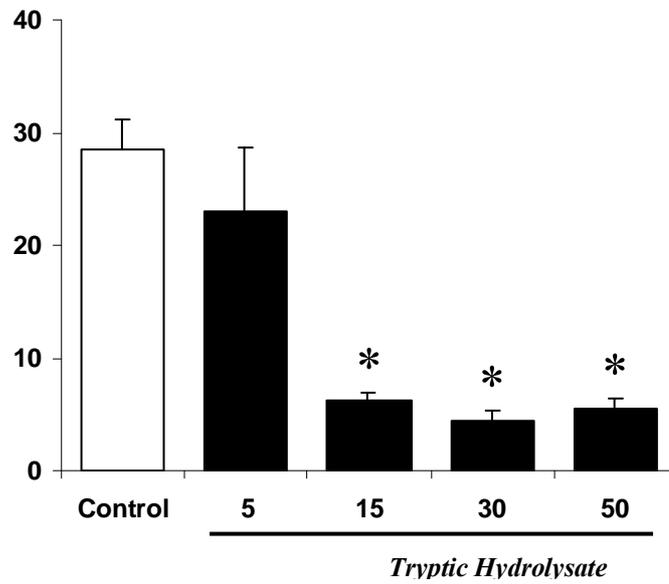
Fig. 1. Dose-response relationship between bovine  $\alpha_{s1}$ -casein tryptic hydrolysate administration (5, 15, 30 and 50 mg/kg, p.o.) and behaviour in the conditioned defensive burying test. Data presented are the probe burying duration (A), the latency of the first approach toward the probe (B), and the latency of the first contact with the probe (C). Results are expressed as mean  $\pm$  SEM.  $n=8$  in each group. \*  $p<0.05$  (Scheffé's test), statistically significant difference from controls.

Fig. 2. Effects of the bovine  $\alpha_{s1}$ -casein tryptic hydrolysate (15 mg/kg, p.o.) and diazepam (3 mg/kg, p.o.) on the probe burying duration (A), the latency of the first approach toward the probe (B), and the latency of the first contact with the probe (C), measured in the conditioned defensive burying test. Results are expressed as mean  $\pm$  SEM.  $n=10$  in each group. \*  $p<0.05$  (Scheffé's test), statistically significant difference from controls.

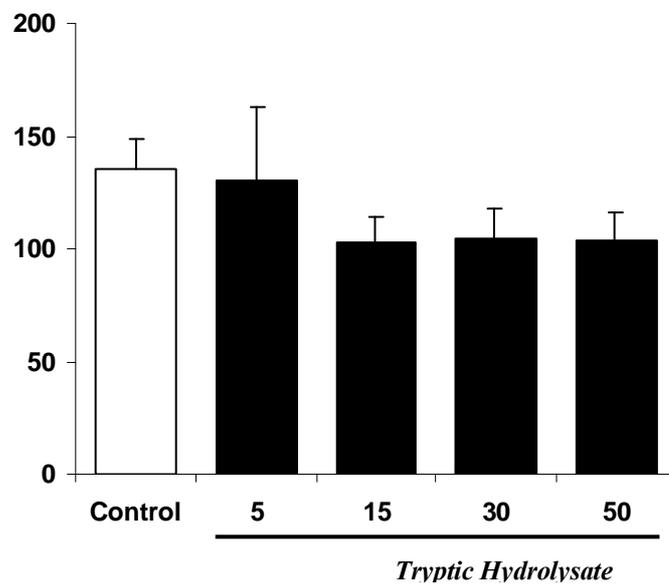
### A. Probe burying duration (s)



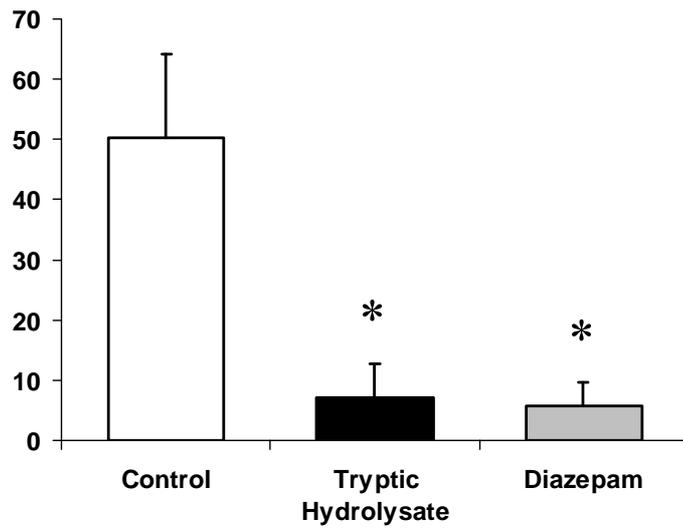
### B. Latency of the first probe approach (s)



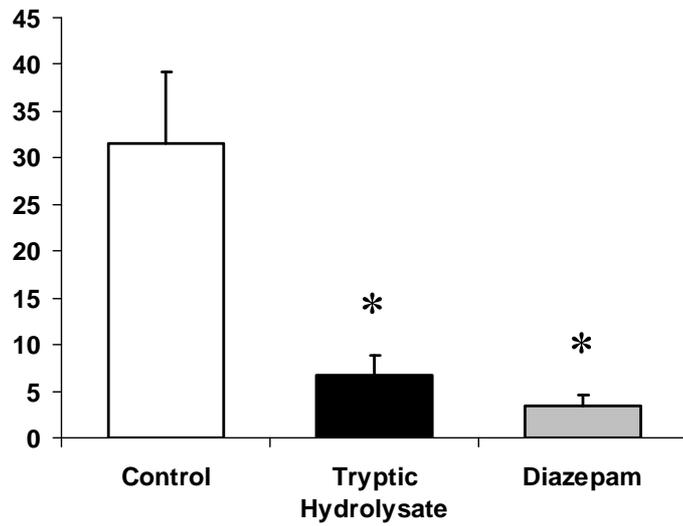
### C. Latency of the first probe contact (s)



### A. Probe burying duration (s)



### B. Latency of the first probe approach (s)



### C. Latency of the first probe contact (s)

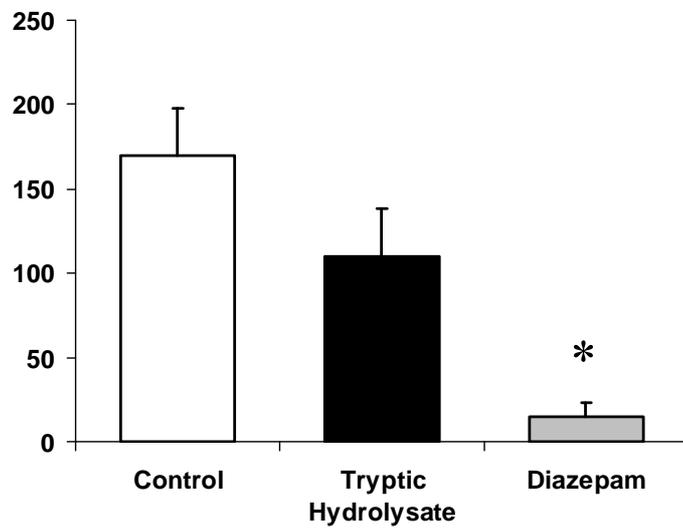


Table 1

Effects of the bovine  $\alpha_{s1}$ -casein tryptic hydrolysate and diazepam on the anxiety-linked behaviours in the elevated plus-maze

Variable	Control	Diazepam 3 mg/kg	Tryptic Hydrolysate 15 mg/kg	ANOVA	
				F(2, 29)	<i>p</i>
Total arm entries	10.0 ± 0.91	15.9 ± 0.90 **	9.9 ± 1.29 #	10.701	<0.01
Percent open arm entries	28.5 ± 5.64	52.8 ± 4.39 *	61.8 ± 6.26 **	9.996	<0.01
Percent closed arm entries	72.2 ± 5.42	46.1 ± 4.77 **	37.0 ± 6.25 **	10.966	<0.01
Percent open arm time	25.2 ± 4.40	52.0 ± 4.60 **	46.8 ± 5.80 *	8.148	<0.01
Total head dips	11.6 ± 2.72	25.3 ± 2.46 *	17.4 ± 2.73	5.803	<0.01
Open arm head dips	6.8 ± 2.10	18.5 ± 2.36**	15.3 ± 2.74	6.255	<0.01
Total rears	23.6 ± 0.72	21.8 ± 3.32	17.0 ± 1.41	2.585	n.s.
Open arm rears	1.4 ± 0.65	4.6 ± 1.04 *	3.0 ± 0.79	3.586	<0.05
Total rearing duration (s)	43.1 ± 3.48	34.1 ± 5.06	30.8 ± 1.20	3.090	n.s.
Open arm rearing duration (s)	1.2 ± 0.54	5.7 ± 1.65 *	2.9 ± 0.77	4.401	<0.05

Results are expressed as mean ± SEM of *n*=10 animals/group. Scheffé's test was used for post-hoc analysis.

n.s. not significant

\* *p*<0.05 statistically significant difference from controls\*\* *p*<0.01 statistically significant difference from controls# *p*<0.05 statistically significant difference from diazepam-treated animals