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The inhibiting effect of Japanese quail egg ovomucoid on human trypsin and elastase activity

Summary:

The egg whites from several avian species contain a glycoprotein known as ovomucoid which presents inhibiting properties against serine proteases. Japanese quail egg white has been used as a dietary supplement in patients suffering from allergic conditions and inflammatory phenomena. Repeated administration of courses of treatment lasting several days involving high doses has enabled their condition to be improved. We wanted to demonstrate the inhibiting potential of this nutrient (which contains ovomucoid) on the activity of serine proteases of human origin. We purified the ovomucoid in 2 stages: precipitation in an acetone/trichloracetic acid medium, followed by protein fractionation on an affinity matrix (heparin-agarose). MALDI-TOF-MS mass spectrometry enabled the nature of the proteins purified to be identified. Then we studied the inhibiting effect of the purified ovomucoid on human and bovine trypsin activity as well as on porcine and human elastase activity. Our results show that purified ovomucoid has an inhibiting effect on both human and bovine trypsin activity and shows a low yet significant inhibition on porcine or human elastase activity. The results obtained suggest a strong specificity of the ovomucoid in terms of its properties for inhibiting the activity catalysed by trypsin. The competitive nature of this inhibition justifies both repeated administration of high-dose courses of treatment involving Japanese quail eggs to patients as a dietary supplement and the improvements observed. A galenic form adapted to use of the purified ovomucoid in the treatment of patients suffering from allergic conditions should contribute to breaking the inflammatory process observed.

Key words: Serine proteases, bovine trypsin, porcine elastase, egg white, allergic conditions

1. Introduction

Japanese quail egg has been used for centuries as a nutritherapy in countries with shortcomings in terms of their pharmacopoeias. In 1978, Truffier reported ten years' worth of observations of his clientele, and particularly regarding patients suffering from allergic conditions and inflammatory phenomena (44). This purely empirical observation was subsequent to courses of treatment involving quail eggs; the patients concerned consumed 5 eggs in the morning while fasting, with sequences lasting several days and with periods where this was interrupted. The observations reported were certainly not clinical study results conducted under double blind conditions, but the number of patients "treated" (around 5,000) with significant improvements in 70% of the cases, was significant. We now know that Japanese quail egg white contains serine protease inhibitors, such as ovomucoid, which are capable of inhibiting the activity of human trypsin. Trypsin is a digestive enzyme produced by the exocrine portion of the pancreas, a minute part of which passes into the blood. The proteolytic activity of trypsin is so powerful that it is, under normal conditions, inhibited by alpha-1 antitrypsin and alpha-2 macroglobulin. This serine protease acts on the immune cells and inflammation by activating specific receptors in the cell membrane (PAR2 or Protease Activated Receptor 2). What ensues is production of superoxide anions by NADPH oxidase (27) associated with intracytoplasmic degranulation (22). Recent studies have shown that most of the allergens inhaled had a "trypsin" type structure (23). The body of an allergic or atopic subject may be invaded by a huge quantity of serine protease type allergens, leading to a break in the proteases/anti-proteases balance (40). The allergen/IgE antibody conflict then leads to degranulation of basophils and mastocytes which simultaneously release the following into the blood: serine proteases, histamine, interleukin 4 and chemotactic factors; elements which contribute to specific allergic inflammation of a given allergen. The inflammatory response observed may be non-specific and endogenic (pancreatic tryspin and neutrophil elastase) or specific and exogenic (trypsin type allergens with serine protease activity).

Protease inhibitors are well represented in nature and play a part in balancing numerous biological functions. It was demonstrated in 1903 that chicken egg white is capable of inhibiting trypsin activity in mammals, with the identity of this protein as an ovomucoid only being established in 1947 (8). Ovomucoid is found in the egg white of numerous species of

birds; it is a stable, specific glycoprotein which has an inhibiting effect in relation to serine proteases like trypsin by forming an enzyme-inhibiting complex. Japanese quail egg ovomucoid, a 28 kDa glycoprotein, consists of three homologous Kazal domains in tandem, each of which has an S1 sequence corresponding to the active or reaction site and a P1 sequence involved in contact and recognition of the serine protease to be inhibited (15). The inhibiting properties of ovomucoids differ depending on the species and, within each species, depending on the protease target (2). Adopting a structural approach, great variability in the residues belonging to contact region P1 was demonstrated and, to the contrary, a high level of preservation in the case of the strategic residues involved in S1 inhibiting activity (12, 15).
Japan quail egg white is used as a dietary supplement. The objective of this work was to demonstrate the inhibiting potential of this nutrient on the activity of human serine proteases. During the initial stage, we developed a method for purifying the ovomucoid, known for its inhibiting activity on serine proteases, based on Japanese quail egg white. During the second stage, we studied the inhibiting power of purified glycoprotein on the activity of two serine proteases, human trypsin and elastase.

2. Material and methods

2.1 Reagents

Heparin agarose, type I H6508; bovine pancreas trypsin, T1426; human pancreas trypsin, T6424; human leukocyte elastase, E8140; porcine pancreas elastase, E1250; BAEE (Nα-Benzoyl-L-Arginine Ethyl Ester Hydrochloride), B4500; N-Succinyl-Ala-Ala-Ala-p-Nitroanilide; S4760, Sigma Chemical Co. (St Louis, CA, USA). Coomassie stain solution, 161-0436, Bio-Rad (Richmond, CA, USA).

2.2. Purification of the ovomucoid

The ovomucoid was purified from egg whites from the Japanese quail (*Coturnix coturnix bemina japonica*). When the eggs are received, the whites are immediately separated from the

yolks and are frozen at a temperature of -20°C. The egg whites are defrosted at room temperature and are gently diluted in 2 volumes of freshly prepared precipitate solution (1 volume of trichloracetic acid 0.5 M and 2 volumes of acetone) (2, 3, 8). Contact maintained by agitating for 12 hours at 4°C. After being centrifuged at 3,000 g for 30 minutes at 4°C, the supernatant is diluted using 2 volumes of cold acetone. The precipitate is recovered by centrifuging it at 3,000 g for 20 minutes at 4°C and is dissolved in a small volume of water. The solution obtained is dialysed against distilled water for one night and is then freeze-dried. The "raw ovomucoid" powder is then recovered and kept at a temperature of 4°C. The raw ovomucoid (100 mg) is dissolved in 50 ml of 5% formic acid solution with a pH of 3 for 2 hours whilst being agitated at room temperature. The whole lot is centrifuged at 500 g for 5 minutes under the same temperature conditions in order to eliminate the undissolved particles. The centrifuged supernatant is then placed in a chromatography column (1.6 x 20 cm) of heparine agarose that has been balanced beforehand using 5% formic acid solution (v/v) with a pH of 3 and subjected to a flow rate of 18 ml per hour (27). 3 ml fractions are recovered and the absorbance at 280 nm is measured. After the filtrate is passed through, the column is washed using a solution of NaCl 0.1 M with a pH of 7.5 until the absorbance reaches its base level, $A_{280} = 0$, and then the proteins are eluted using a discontinuous gradient of NaCl 0.5 M and then 1 M, with a pH of 7.5. The proteins contained in the eluates are fractionated by electrophoresis on 12.5 % polyacrylamide gel followed by dying it using Coomassie blue. The interesting fractions are brought together, diluted into 2 volumes of distilled water, and are then freeze-dried and stored at 4°C.

2.3. Identification of proteins using MALDI-TOF-MS

The proteins which were purified beforehand using chromatography, were identified using the MALDI-TOF-MS (Matrix-Assisted Laser Desorption Ionisation – Time Of Flight Mass Spectrometry) (28, 29). 10 % SDS-PAGE electrophoresis with a thickness of 0.75 mm was carried out by depositing 10 µl of each of the fractions containing the proteins to be analysed. Dying them Coomassie blue was carried out by fixing the proteins in 2 baths of 7.5 % acetic acid/30 % ethanol, each lasting a minimum of 30 minutes, and then for 60 minutes in the Coomassie blue dye solution, followed by several dye-removal baths using 30 % ethanol until an optimal contrast was achieved. The proteins of interest were excised from the gel dyed Coomassie blue and were then digested using the technique described earlier (28). The proteins

were extracted from the piece of gel using a solution containing 5 % formic acid (v/v) and acetonitril. Afterwards, the tryptic peptides are dried and resuspended in a 5 % aqueous solution of trifluoroacetic acid. The samples are preconcentrated (preliminary column: 300 µm x 5 mm by PepMap C18) before being injected into the LC-Packings (Dionex) nanoLC system. The peptides are eluted in a C18 column (75 □m x 150 mm). The chromatographic separation is carried out using a gradient of solution A (5 % acetonitril; 95 % water; 0.1 % formic acid) in solvent B (CH_3CH/Water 90/10 (v/v) containing 0.1 % FA) for 60 minutes at a flow rate of 200 nL per minute. The LC system is directly linked to a QTOF Ultima mass spectrometer (Waters). The raw data were processed using PeptideAuto software (ProteinLynx, MassLynx 4.0). The data search was carried out using the MASCOT search engine (Intranet 2.1, MASCOT software version) in relation to the Swissprot Trembl database. For identifying the proteins for peptides with a score higher than 40, the peptide sequence was examined manually. For identifying proteins with a score higher than 20 and lower than 40, the peptide sequence was examined manually and/or interpreted manually in order to either confirm or rule out the suggestion made by the MASCOT software. The remaining peptides were identified manually using the MS-Pattern (http://prospector.ucsf.edu/) and Blast (http://www.ncbi.nlm.nih.gov/BLAST/) database sites.

2.4. Trypsin activity dosage

The E.3.4.21.4 trypsin activity (bovine or human) is measured using a spectrophotometry method utilising BAEE (Benzoyl-L-Arginine Ethyl Ester) as a substrate (13, 14). A pre-incubation medium was prepared by adding 100 µl of bovine trypsin at a rate of 0.3 mg/ml or human trypsin at a rate of 0.5 mg/ml in HcL 1 mM, with volumes increasing from 0 to 50 µl of purified ovomucoid purified at a rate of 2 mg/ml. The medium was then topped up to 1 ml using the 67 mM phosphate buffer with a pH of 7.6. The whole lot was incubated at 37°C for 30 minutes. After incubation, the reaction was initiated by adding the substrate, 950 µl BAEE 0.25 mM, to the 67 mM phosphate buffer with a pH of 7.6, to which 50 µl of pre-incubation medium was added. The absorbance at 253 nm of the benzoyl-L-arginine from hydrolysis of the BAEE is then monitored every 30 seconds for 5 minutes. The activity is expressed in mmol of benzoyl-L-arginine/l/µl of protein (trypsin).

2.5. Elastase activity dosage

The E.3.4.21.36 elastase activity is quantified using a spectrophotometry method by measuring the absorbance at 410 nm of the p-Nitroaniline from hydrolysis of the elastase substrate: $SucAla_3$ (N-Succinyl-Ala-Ala-Ala-p-Nitroanilide) (12, 17, 18, 19). In a spectrophotometry tank, 20 µl of elastase at a rate of 1 mg/ml is added to HCl 1 mM, with volumes from 0 to 50 µl of ovomucoid purified at a rate of 2 mg/ml. The medium was then topped up to 1 ml using the 67 mM phosphate buffer with a pH of 7.6. with the whole lot being incubated at 37°C for 30 minutes. The reaction was initiated by adding 40 µl of substrate, with $SucAla_3$-p-Nitroanilide at a rate of 2 mg/ml in the 67 mM phosphate buffer with a pH of 7.6. The absorbance was then monitored at 410 nm every 30 seconds for 10 minutes. The activity is expressed in mmol of $SucAla_3$/l/µg of protein (porcine elastase) or in µmol $SucAla_3$/l/µg of protein (human elastase).

2.6. Electrophoresis in polyacrylamide gel, SDS-PAGE

The proteins are separated using electrophoresis in denaturing gel with 12.5 % polyacrylamide (29). Volumes of 50 µl are used for each fraction obtained via chromatography. After electrophoresis, the gel is incubated for 30 minutes in a 0.25 % R250 Coomassie blue solution in 50 % methanol (v/v) and 10 % acetic acid (v/v), then the dye is removed in a solution of 8 % acetic acid (v/v) and 27 % ethanol (v/v).

3. Results

3.1. Purification of the ovomucoid and identification

The Japanese quail (*Coturnix coturnix bemina japonica*) egg white, stored at -20°C, came from a single egg (approximately 5.5 ml) and was defrosted at room temperature and diluted in 2 volumes of trichloracetic acid/acetone mix (1v/2v) prepared extemporaneously; after 12 hours of incubation at 4°C and dilution in frozen acetone, with the centrifugate being washed and dissolved in a small volume of distilled, freeze-dried water (see Material and Methods). This fraction is the raw ovomucoid which will then be purified on the heparin agarose affinity matrix. Elution using a discontinuous gradient of NaCl enables the 3 fractions to be identified

(Fig. 1A): the first relates to the filtrate and features a mix of proteins which are not fixed on the matrix, with one of them being predominant with a molecular weight of 45 kDa (Fig. 1B). The protein content of the 2 elution fractions using NaCl 0.5 M and NaCl 1 M is analysed via SDS-PAGE (Fig. 1B): we observed two bands, at 35 kDa and 67 kDa respectively.

MALDI-TOF-MS mass spectrometry enabled the nature of the content of each of these fractions (Table 1) to be identified. The main protein in the filtrate is a protein that is approximately 45 kDa (Fig. 1B), identified as being from the ovomucoid. This ovomucoid could relate to a precursor which is not fixed on the matrix (16). The elution fraction by NaCl 0.5 M contains a 35 kDa protein according to SDS-PAGE (Fig. 1B), a protein which, using the MALDI-TOF-MS technique, was identified as being from the ovomucoid. To the contrary, the fraction eluted with the NaCl 1 M solution contains a 67 kDa protein which has not yet been identified but is not from the ovomucoid. The fractions of interest are then put together, diluted in 2 volumes of distilled water, and are then freeze-dried and stored at 4°C until such time as they are used.

3.2 The inhibiting effect of the ovomucoid on trypsin activity

The inhibiting effect of purified Japanese quail egg ovomucoid on heparin agarose, a 35 kDa protein, was tested on either bovine (Fig. 2) or human (Fig. 3) trypsin activity. Increasing concentrations of purified ovomucoid added to the reaction medium containing a fixed quantity of bovine trypsin (1.5 µg) have an inhibiting effect on trypsin activity, which increases with the quantity of inhibiting protein added (Fig. 2A). We noticed that the effect peaks during the first two minutes of incubation (Fig. 2B). This effect is inversely proportional to the incubation time (Fig. 2A); indeed above and beyond the 2nd minute of incubation, the inhibiting capacity decreases (Fig. 2A). We obtain 100 % inhibition after incubation of 1.5 µg of bovine trypsin for 5 minutes, with 3 µg of purified ovomucoid (1 to 2 ratio) (Fig. 2C). The effects on human trypsin are similar (Fig. 3) but the inhibiting capacity of the ovomucoid at the concentrations used is largely inferior. You have to incubate 10 µg of purified ovomucoid with 2.5 µg of human trypsin (1 to 4 ratio) for only 1 minute for complete inhibition to occur (Fig. 3C); if you increase the incubation time, the trypsin activity will be restored, although even slower than the rate of increase in the ovomucoid concentrations.

3.3 The inhibiting effect of the ovomucoid on elastase activity

The inhibiting effect of purified Japanese quail egg ovomucoid was tested on porcine elastase activity (Fig. 4) and then on human elastase activity (Fig. 5). Increasing concentrations of ovomucoid added to the reaction medium containing a fixed quantity of porcine elastase (20 µg) led to low-level inhibition which increased with the quantity of inhibiting protein added (Fig. 4A). We thus noticed that inhibition of porcine elastase activity (20 µg) by the purified ovomucoid reaches a maximum level of 30 % and that you have to incubate 100 µg of ovomucoid for 10 minutes with 20 µg of elastase in order to obtain maximum inhibition (Fig. 2B), which represents an inhibiting enzyme ratio of 1 to 5. The inhibiting effect of the purified ovomucoid on human elastase activity is much more marked with an optimum achieved for 10 µg of ovomucoid incubated for 2 minutes with 20 µg of human elastase. This inhibiting effect (25 %) is proportional to the incubation time. When we incubate 20 µg of human elastase with 100 µg of purified ovomucoid, with a ratio of 1 to 5 for 10 minutes, the inhibition percentage obtained is approximately 60-70%.

4. Discussion

Numerous serine protease inhibitors of various origins are currently known (1, 2, 4, 6, 11). The egg whites from several avian species contain a glycoprotein known as ovomucoid which has inhibiting properties in relation to serine proteases, properties which vary depending on the origin of these enzymes (3-9, 11, 16). More particularly, these inhibiting properties are targeted at bovine trypsin activity, bovine alpha-chymotrypsin, and subtilisin (6). In our work, we were interested in the ovomucoid from Japanese quail (*Coturnix coturnix bemina japonica*) egg white.

We purified the ovomucoid in 2 stages: precipitation in an acetone/trichloracetic acid medium followed by protein fractionation on an affinity matrix (heparin agarose) which shows a preference for fixing glycoproteins (27). Just one protein, identified as being the ovomucoid, was eluted from the heparin matrix using a solution of NaCl 0.5 M. Its molecular weight, 35 kDa, differs from that of the ovomucoid (28 kDa) previously described in Japanese quail eggs (3, 16). The more or less glycosylated nature of these 2 proteins could provide the explanation

for these differences. Furthermore, the presence of a 45 kDa molecule may be observed in the fraction which contains the proteins not retained on the affinity matrix. An ovomucoid precursor has been described; it includes 23 additional amino acids at the N-terminal end, close to certain signal peptides (16): the 45 kDa protein could relate to this precursor (Table 1).

We confirmed that the ovomucoid purified from Japanese quail egg whites is an inhibitor of bovine tryspin, which has been described previously (2, 6) and demonstrated a low yet significant inhibition with porcine or human elastase. We did however observe that the inhibiting capacity of the purified ovomucoid varies depending on the origin of the trypsin: subject to the same conditions for incubating and measuring trypsin activity, the inhibiting effect of the purified ovomucoid is lower with human trypsin (60 %; a 1 to 4 enzyme/inhibitor ratio) compared to what is observed with bovine enzyme (100 %; 1 to 2 ratio).
The inhibition properties of the ovomucoid vary depending on the origin of the trypsin and depending on the nature of the serine protease targeted. The ovomucoid does in fact inhibit elastase to a very low level whether it is of porcine or human origin, which is an argument in favour of its specificity for trypsin (15, 3, 2, 4, 5, 6). The ovomucoid is a competitive inhibitor. This property, which has already been reported (26) is illustrated in Figures 2A and 2B. The ovomucoid and the substrate bond at the enzyme's active site: competition between the ovomucoid and the substrate observed *in vitro* in the reaction catalysed by trypsin depends on the concentration of the elements present and the incubation time. Contact is therefore the specific recognition feature between the inhibitor (ovomucoid) and the serine protease depending on the nature of the amino acid residues at the ovomucoid's P1 site (3, 12, 15, 25). The great variability in these residues depending on the purified ovomucoid sources could provide an explanation for the different inhibition rates reported in the literature (4, 6, 7). In the catalysis mechanism, good alignment of the strategic residues at the active site of the enzyme (trypsin or elastase) with groupings of the substrate precondition the catalysed activity, the catalysis speed (activity) and, therefore, the specificity, with it being linked to the nature of the cleaved peptide sequence. In the inhibition observed with the ovomucoid, competition between the enzyme and the inhibitor leads to a reduction in the enzyme's affinity for its substrate.

5. Conclusion

The results obtained suggest that ovomucoid has a high specificity in terms of its inhibition properties in relation to the activity catalysed by trypsin. The competitive nature of this inhibition justifies both repeated administration of the high-dose courses of treatment involving Japanese quail eggs to patients as a dietary supplement and the improvements observed. A galenic form adapted to the use of purified ovumucoid in the treatment of patients suffering from allergic conditions should contribute to breaking the inflammatory process observed.

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Table 1: Identification of purified proteins from quail eggs using the MALDI-TOF-MS technique. After trypsic digestion of the proteins, which were purified beforehand in a column of heparin agarose with electrophoresis migration, the fragments obtained were identified using MALDI-TOF-MS mass spectrometry. For each fragment, the mass is determined, along with the sequence, and then comparison with the databanks enables a known homologous sequence to be defined.

Protein	Mass	Sequence	Homologue
30 kDa	601.3 896.41 1,079.46	KKRVSDGTLTLNHFGK NNQPAHNLVQGTSVGKK LEAAVSVDCSEYPMMVPLR	sp|P52264|IOVO_PODST Ovomucoid (Fragment) sp|P01003|IOVO_COTJA Ovomucoid sp|P67944|IOVO_TYMCU Ovomucoid (Fragment)
45 kDa	690.97 697.3 889.4	...GPADYRPVCGSDNK (MLQ)GVVCPDDLR ..QPAHNVVQGTSVGKK	sp|P01003|IOVO_COTJA Ovomucoid sp|P01003|IOVO_COTJA Ovomucoid sp|P01003|IOVO_COTJA Ovomucoid
60 kDa	574.01 598.69 613.27 796.81	...AAAALSHLVCGVPR ..LLLPRCRVLGGAHK ..YDVTCSAAPPR ..VDCSQAHASGLSK	- - - -

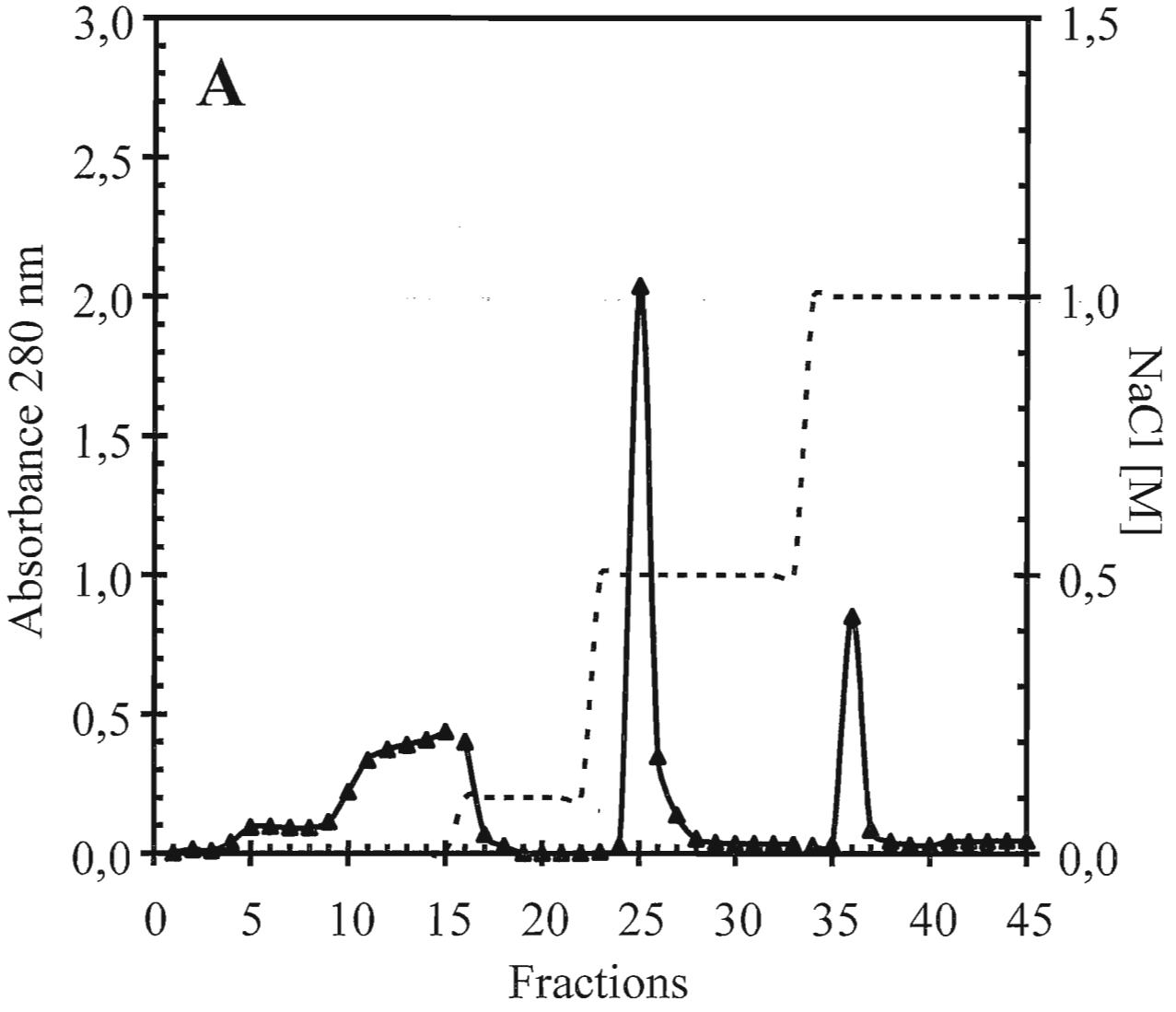
Fig. 1. Purification of the ovomucoid. (A) The raw ovomucoid (RO) is obtained from quail egg white after precipitation using a trichloracetic acid-acetone solution. 100 mg of raw ovomucoid is deposited on the heparin agarose matrix. The filtrate is recovered (fractions 1 to 14), and the column is washed using NaCl 0.1 M (fractions 15 to 23) and then the proteins fixed on the column are eluted using NaCl 0.5 M (fractions 24 to 33) and then NaCl 1 M (fractions 34 to 35). (B) The elution fractions (50 µl) are checked using electrophoresis in polyacrylamide gel (Coomassie blue dye).

Fig. 2. Inhibition of bovine trypsin activity by the ovomucoid. Bovine trypsin (1.5 µg) is incubated in the presence of 0.25 mM of BAEE (N-Benzoyl-Arginine-Ethyl-Ester) in a 67 mM phosphate buffer with a pH of 7. The activity is measured at 37°C every 30 seconds for 5 minutes whilst monitoring absorbance at 253 nm; it is expressed in mmol BAEE/l/µg of bovine trypsin. (A-B) The effect of increasing quantities of purified ovomucoid (0 to 3 µg) on bovine trypsin activity (1.5 µg) depending on the time (n = 5). (C) Inhibition percentage by the ovomucoid on bovine trypsin activity at various incubation times (n = 5).

Fig. 3. Inhibition of human trypsin activity by the ovomucoid. Human trypsin (2.5 µg) is incubated in the presence of 0.25 mM of BAEE (N-Benzoyl-Arginine-Ethyl-Ester) in a 67 mM phosphate buffer with a pH of 7. The activity is measured at 37°C every 30 seconds for 5 minutes whilst monitoring absorbance at 253 nm; it is expressed in mmol BAEE/l/µg of human trypsin. (A-B) The effect of increasing quantities of purified ovomucoid (0 to 25 µg) on human trypsin activity (2.5 µg) depending on the time (n = 5). (C) Inhibition percentage by the ovomucoid on bovine trypsin activity at various incubation times (n = 4).

Fig. 4. Inhibition of porcine elastase activity by the ovomucoid. Porcine elastase (20 µg) is incubated in the presence of N-Succinyl-Ala-Ala-Ala-p-Nitroanilide (80 µg) in a 67 mM phosphate buffer with a pH of 7.6. The activity is measured at 37°C every 30 seconds for 10 minutes whilst monitoring absorbance at 410 nm; it is expressed in µmol $SucAla_3$/l/µg of porcine elastase. (A) The effect of increasing quantities of purified ovomucoid (0 to 140 µg) on porcine elastase activity (20 µg) depending on the time (n = 5). (B) Inhibition percentage by the ovomucoid on porcine elastase activity at various incubation times (n = 5).

Fig. 5. Inhibition of human elastase activity by the ovomucoid. Human elastase (20 µg) is incubated in the presence of N-Succinyl-Ala-Ala-Ala-p-Nitroanilide (80 µg) in a 67 mM phosphate buffer with a pH of 7.6. The activity is measured at 37°C every 30 seconds for 10 minutes whilst monitoring absorbance at 410 nm; it is expressed in µmol $SucAla_3$/l/µg of human elastase (20 µg). (A) The effect of increasing quantities of purified ovomucoid (0 to 50 µg) on human elastase activity (20 µg) depending on the time. (B) Inhibition percentage by the ovomucoid on porcine elastase activity at various incubation times (n = 5).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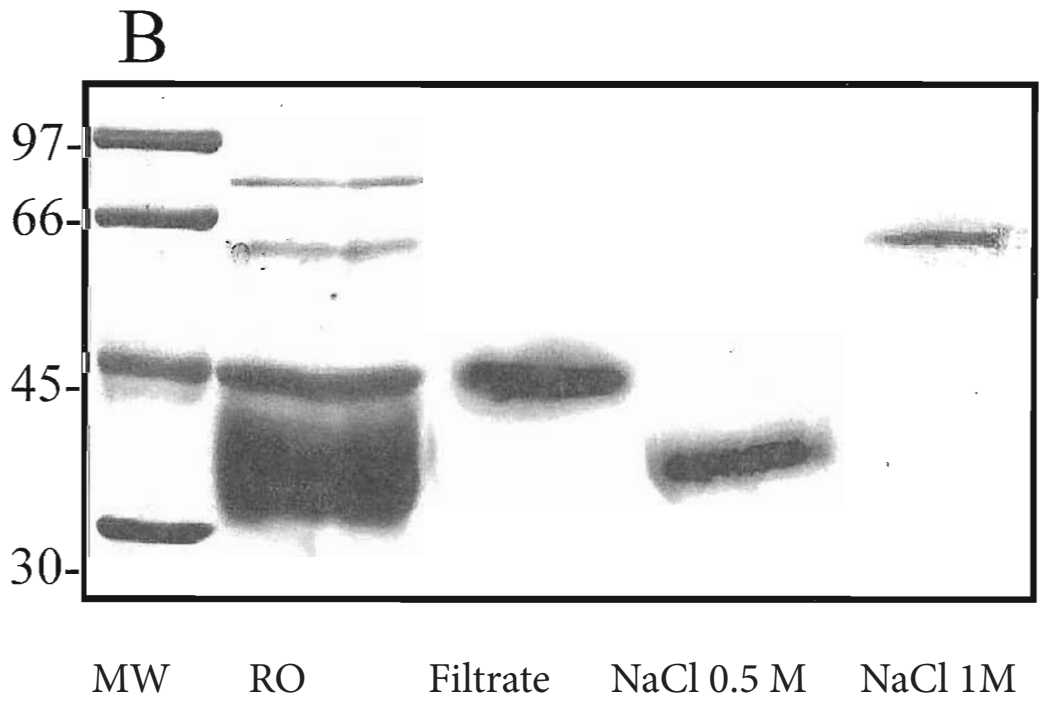
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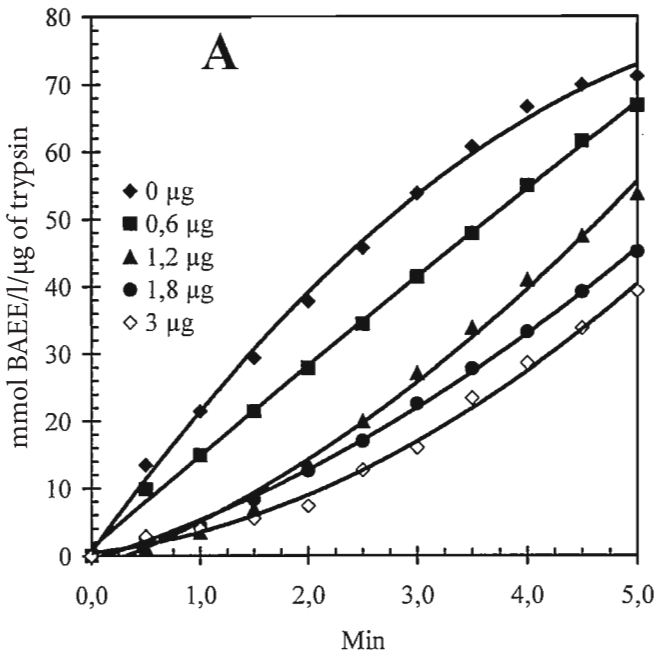
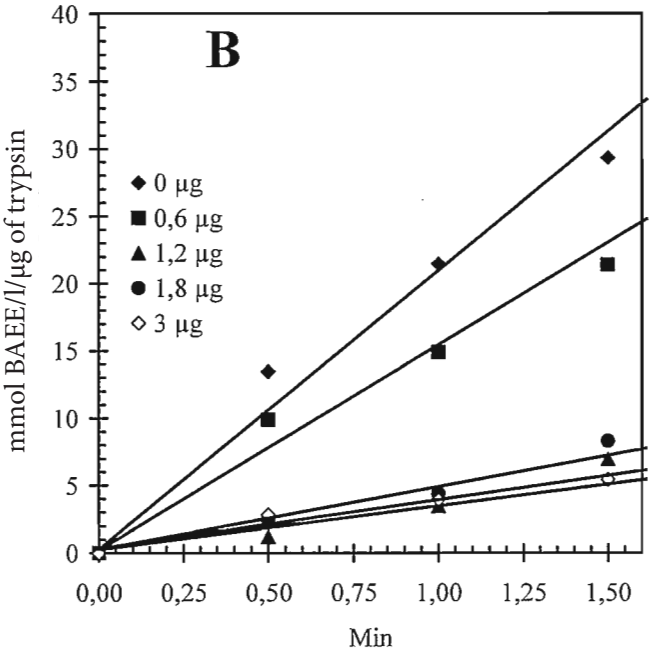
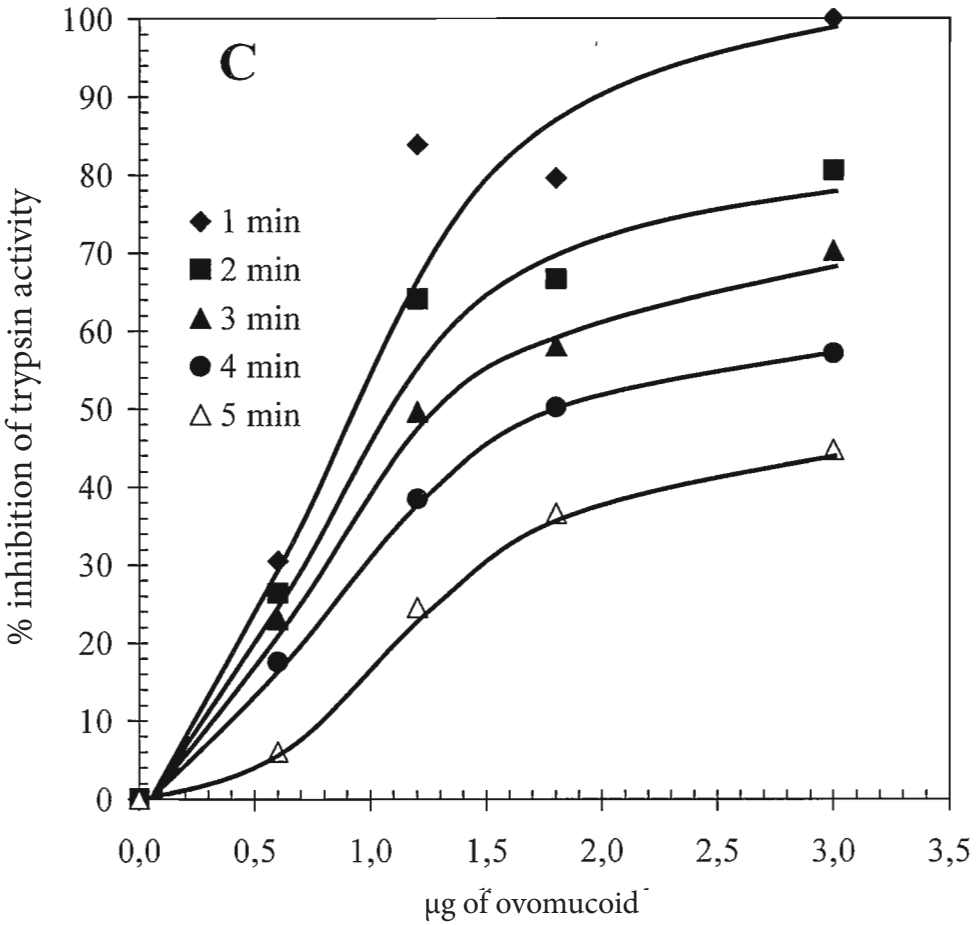
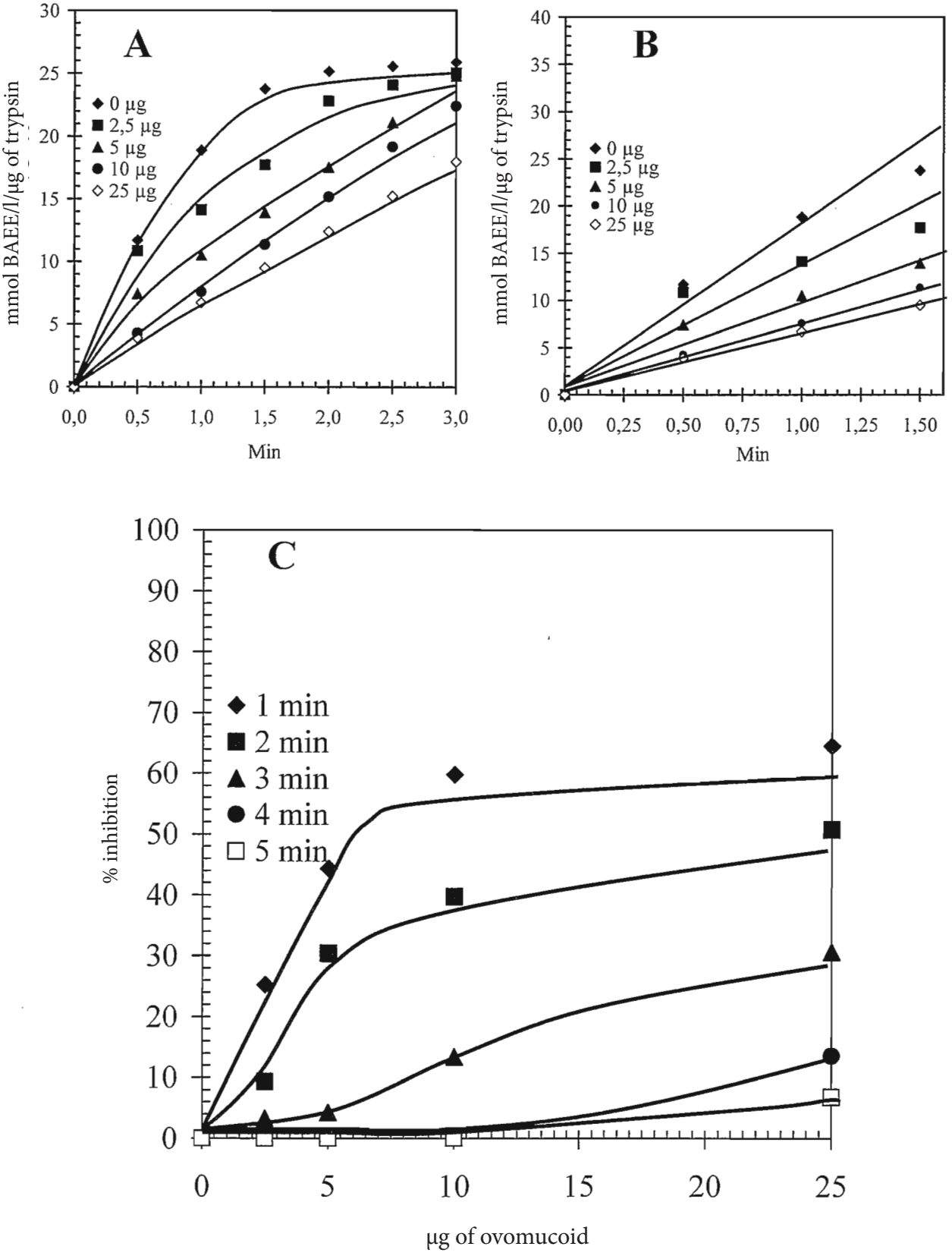
Bovine Trypsin 1.5 µg



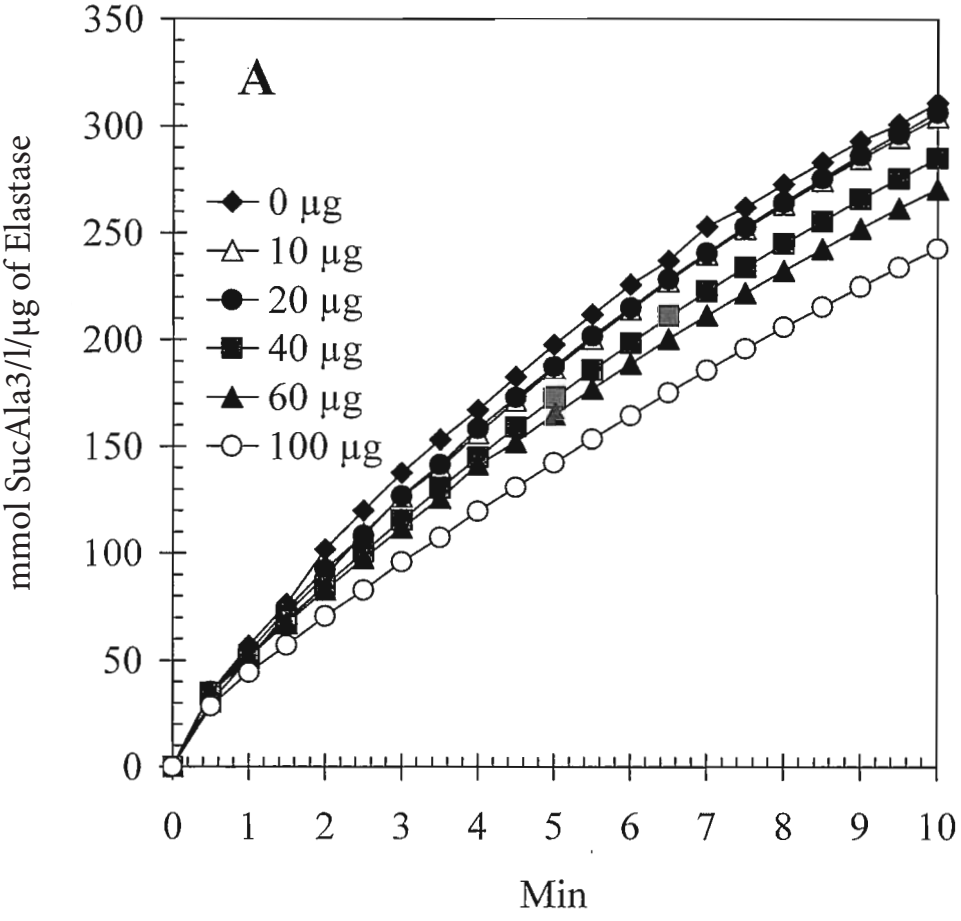


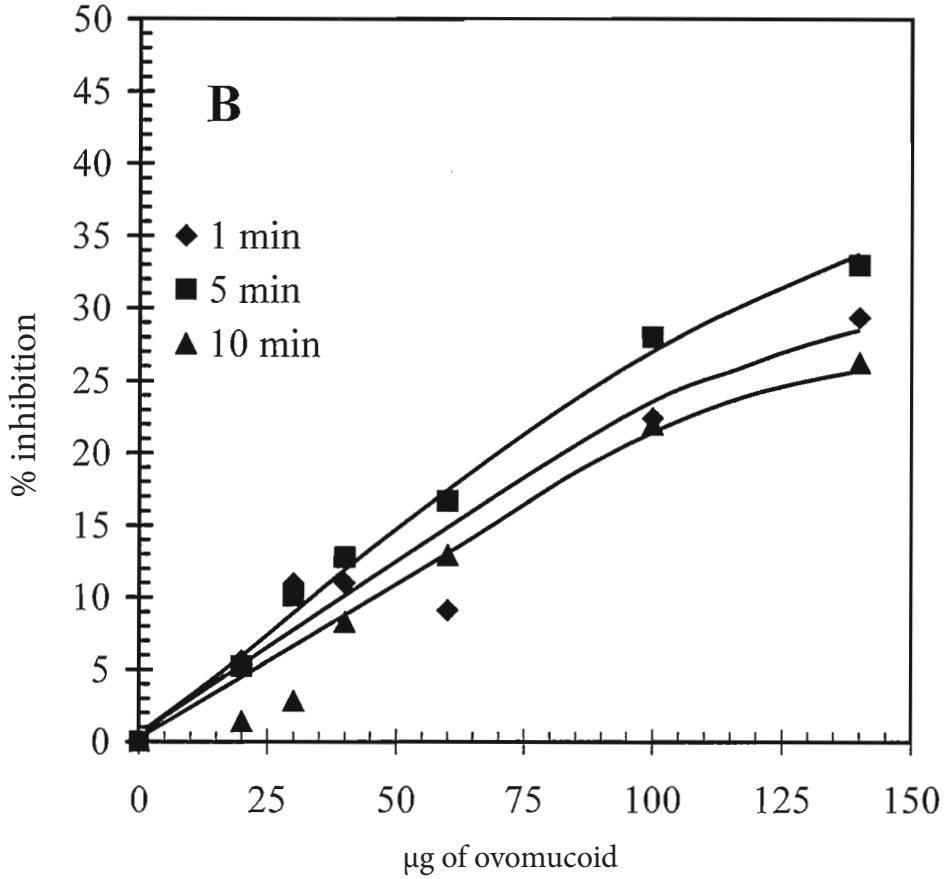


Human Trypsin 2.5 µg



Porcine Elastase 20 µg





Human Elastase 20 µg