

## Peptides affecting coagulation

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Based on amino acid sequence similarities that exist between the fibrinogen  $\gamma$ -chain and  $\kappa$ -casein, and also functional similarities between milk and blood coagulation, considerable effort has been made to investigate the effects of milk proteins and peptides on platelet function and thrombosis. In particular, a number of peptides derived from the glycomacropeptide segment of  $\kappa$ -casein, have been shown to inhibit platelet aggregation and thrombosis. KRDS, a peptide from lactoferrin has also been shown to inhibit platelet aggregation but to a lesser extent than its fibrinogen analogue RGDS. Despite their functional and structural similarities they do not act in the same way on platelet function and are thought to affect thrombus formation differently. Further investigation is needed to determine if these milk-derived bioactive peptides are released naturally following ingestion and might therefore be useful as the basis for milk-based products with anti-thrombotic properties.

### Bioactive peptides: Blood coagulation

#### Introduction

Milk contains a large number of bioactive peptides that either exist naturally or can be released via enzymatic proteolysis of the parent milk proteins. These peptides have various biological activities. Some peptides have a marked influence on gastrointestinal functions, whereas others, such as immunostimulating peptides, antihypertensive peptides (ACE inhibitors) and peptides affecting blood coagulation have significant effects on general health. The production of bioactive peptides derived from digestion of milk proteins, or during food processing, may therefore prove to be physiologically important to both the newborn and to adults.

#### Comparison between blood and milk clotting processes

Blood clotting is the most important defence mechanism for preventing blood loss following vessel or tissue damage. Platelet aggregation is the first and most important step of this process. Platelet attachment, spreading and aggregation on extracellular matrices are central events in thrombus formation. Platelet glycoprotein GPIb is essential for the initial adhesion of platelets to the exposed subendothelium, an event that is followed by the binding of fibrinogen to platelet GPIIb/IIIa.

Blood clotting and milk clotting are both important physiological coagulation processes. There are broad similarities between blood and milk clotting processes, and these include sequence identities between fibrinogen

and some milk proteins, and also some functional similarities.

The human fibrinogen  $\gamma$ -chain displays some sequence similarity to bovine  $\kappa$ -casein or its glycomacropeptide (GMP). In fact Jolles *et al.* (1978) hypothesized that fibrinogen  $\gamma$ -chain and  $\kappa$ -casein may have evolved from a common ancestor during the past 450 million years. There are structural and functional similarities between the fibrinogen  $\gamma$ -chain C-terminal dodecapeptide (400–411), which is involved in binding to platelet receptors, and various peptides from the 106–116 region of bovine  $\kappa$ -casein (Table 1), which are termed casoplatelins. Similarities also exist between the fibrinogen  $\alpha$ -chain tetrapeptide (RGDX) and human lactoferrin (KRDS, residues 39–42). Both have a high probability of initiating a  $\beta$ -turn and are highly hydrophobic.

The fibrinogen cleavage actions of the blood clotting enzyme thrombin and the  $\kappa$ -casein cleavage actions of the milk clotting enzyme chymosin also bear some similarities. Both the blood and milk clotting processes involve limited proteolysis; thrombin cleaves two R-G bonds to produce fibrin and fibrinopeptides, whereas chymosin cleaves a single unique F-M bond to form para- $\kappa$ -casein and GMP. Short soluble peptides (fibrinopeptides and caseinoglycopeptides) are released during both blood and milk coagulation processes. Both of the released peptides are highly variable in sequence yet maintain a net negative charge, and neither contains cysteine or tryptophan residues. The  $\epsilon$ -amino groups of lysine appear to be involved in the polymerisation of both fibrin and casein,

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**Table 1.** Comparison of amino acid sequences of fibrinogen and a peptide from bovine  $\kappa$ -casein

Fibrinogen $\gamma$ -chain dodecapeptide	<sup>400</sup> H H L G G A K Q A G D V <sup>411</sup>
$\kappa$ -casein undecapeptide	<sup>106</sup> M A I P P K K N Q - D K <sup>116</sup>
Fibrinogen $\gamma$ -chain	<sup>169</sup> I K P L K A N Q Q F <sup>177</sup>

and calcium is important in both processes, accelerating the second phase of milk clotting and the aggregation of fibrin monomers. Prosthetic sugar groups do not play an important role in the clotting processes, however they retard the rate of chymosin or thrombin action.

### Antithrombotic activity of $\kappa$ -casein and glycomacropeptide

Whole  $\kappa$ -casein inhibits thrombin-induced platelet aggregation and thrombin-induced secretion of serotonin *in vitro*, resulting in 50 % inhibition at 10  $\mu$ M (Drouet *et al.* 1990b). In contrast para- $\kappa$ -casein is inactive in all assay systems. GMP (106–116) inhibits both thrombin and ADP-induced platelet aggregation, causing 50 % inhibition at 10  $\mu$ M and 250  $\mu$ M, respectively. In an *in vivo* rat model experiment, where a targeted laser beam was used to produce a lesion in the endothelium of a mesenteric artery, intravenous injection of GMP led to a 65 % inhibition of thrombogenesis (Drouet *et al.* 1990b). Various vessels were observed for 15 min each, during which the number of thrombi formed was counted. Maximum inhibition of thrombosis was seen during the first 20 min after intravenous injection, with 1.4 thrombi/15 min compared with 4 thrombi/15 min in the control. The effect remained significant until 90 min after the injection of GMP. Similar effects were seen in guinea pigs. These activities have been associated with an undecapeptide sequence (106–116) in the N-terminal region of GMP, which exhibits both structural and functional homology with the C-terminal dodecapeptide (400–411) of fibrinogen  $\gamma$ -chain (Table 1) (Jolles *et al.* 1986, 1993; Maubois *et al.* 1991; Caen *et al.* 1992).

Natural and synthetic peptides from this region of bovine GMP have been shown to inhibit human platelet aggregation and fibrinogen binding *in vitro* to a greater extent than the dodecapeptide of gamma chain fibrinogen (Jolles *et al.* 1986, 1993). Various natural (tryptic digest of GMP) or synthetic peptides from this region (2–10 residue peptides including 106–116, 106–112, 113–116) show a concentration-dependent inhibitory activity to a greater or lesser extent. The natural *in vitro* fragment 106–116 was a strong competitor of ADP-induced platelet aggregation and <sup>125</sup>I-labelled fibrinogen binding to ADP-activated platelets, and was the most active peptide in this study (Caen *et al.* 1992; Jolles *et al.* 1993). It had a more powerful anti-aggregating action on human platelets than the dodecapeptide of fibrinogen. Two smaller peptides (106–112 and 113–116) and a larger peptide (103–111) had less effect on platelet aggregation and did not inhibit fibrinogen binding (Jolles *et al.* 1993).

The minimum effective peptide that was able to inhibit

platelet aggregation and fibrinogen binding to ADP-activated platelets was a pentapeptide generated by tryptic hydrolysis of GMP (112–116) (Jolles & Caen, 1991), which was 30 and 200 times more active, respectively, than peptides 106–116 and 113–116 described by Jolles *et al.* (1986).

The C-terminal caseinoglycopeptides (CGP) (residues 106–171) of ovine  $\kappa$ -casein inhibit thrombin and collagen-induced platelet aggregation in a dose-dependent manner (IC<sub>50</sub> = 215  $\mu$ M and 100  $\mu$ M, respectively). Reverse-phase high performance liquid chromatography fractionation of a trypsin hydrolysate of CGP was found by Qian *et al.* (1995) to result in three distinct peptides; KDQDK (residues 112–116), TAQVTSTEV (residues 163–171) and QVTSTEV (residues 165–171). Each of these peptides completely inhibited thrombin-induced platelet aggregation. Residues 163–171 and 165–171 are situated at the C-terminal end of sheep CGP and as yet no corresponding bovine peptide with similar function has been identified (Qian *et al.* 1995).

Inhibition of platelet aggregation *in vitro* appears to require a very specific structural conformation. For example GMP-derived peptides 106–112 and 113–116 (tryptic digest) have much lower inhibitory activity than the complete fragment 106–116 (Maubois & Leonil, 1989). The interaction appears to be reinforced by the presence of a lysine residue in the sequence; the 112–116 peptide resulting from the trypsin hydrolysis of GMP was 222-fold more active than the 113–116 sequence (Maubois *et al.* 1991). Much of this activity has been measured *in vitro* against blood platelet function, which does not necessarily imply antithrombotic activity *in vivo*. However, *in vivo* antithrombotic activity has been shown for the casein undecapeptide using a laser-induced arterial thrombosis model in rats and guinea pigs. Casein peptides injected by i.v. bolus inhibited thrombus formation in this model (Maubois *et al.* 1991).

The antithrombotic bioactivity of GMP *in vitro* was found to be stable for 1 hour in plasma at 37°C, following which there was a slow degradation, resulting in a 15 % decrease at 75 min and a 30 % decrease at 90 min. GMP showed a 50 % decrease in bioactivity 5 min after i.v. administration, however, GMP was still detectable by HPLC at 90 min in rat plasma (Drouet *et al.* 1990b).

### Activity of lactoferrin and lactoferrin-derived peptides

Human lactoferrin injected as an intact molecule was found to have a minimal effect of short duration on the formation of thrombi *in vivo*, when using an experimental animal model of laser-induced arterial thrombosis (Bal dit Sollier *et al.* 1990). Research with peptide analogues derived from the  $\alpha$ -chain of fibrinogen (RGDS, 572–575) and human lactoferrin (KRDS, 39–42) have all been based on synthetic peptides with identical sequences. KRDS of human lactoferrin inhibited ADP-induced platelet aggregation (IC<sub>50</sub> = 350  $\mu$ M) *in vivo* to a lesser extent than its fibrinogen analogue RGDS (IC<sub>50</sub> = 75  $\mu$ M) (Drouet *et al.* 1990a; Jolles *et al.* 1993). The mean fibrinogen binding (IC<sub>50</sub> = 360  $\mu$ M) of KRDS was lower than that of RGDS (IC<sub>50</sub> = 20  $\mu$ M) (Jolles *et al.* 1993).

Some endogenous peptides are known to inhibit platelet

aggregation. The tetrapeptide RGDS is a sequence that was originally identified as a cell attachment site in fibronectin, it is also present in the structure of fibrinogen and in von Willebrand factor. Synthetic peptides that contain the RGDS sequence inhibit fibrinogen binding and platelet aggregation. Results from studies with synthetic analogues of fibrinogen  $\alpha$ -chain tetrapeptide RGDX and human lactoferrin (KRDS, residues 39–42) show that although KRDS and RGDS have structural and functional similarities, they do not act in the same way on platelet function and therefore probably affect thrombus formation differently (Jolles *et al.* 1986).

KRDS inhibition of thrombin-induced platelet aggregation has been associated with an inhibition of the release of the dense granule protein serotonin, but RGDS had no effect on the release (Drouet *et al.* 1990a). In normal human platelets, thrombin-induced serotonin release was inhibited  $55 \pm 10\%$  by KRDS at  $750 \mu\text{M}$  (Jolles *et al.* 1993). KRDS also inhibited the serotonin release reaction by  $43 \pm 1\%$  in platelets from Glanzmann's thrombasthenia patients, suggesting that the inhibitor pathway is GPIIb/IIIa-independent (Drouet *et al.* 1990a). KRDS inhibits serotonin release by a mechanism independent of protein phosphorylation and thus inhibits fibrinogen binding, and hence aggregation, by a mechanism that may not necessarily involve its direct binding to the GPIIb/IIIa complex (Jolles *et al.* 1993). KRDS did not affect monoclonal antibody (PAC-1) binding to thrombin-activated platelets, whereas in contrast RGDS completely inhibited it (Drouet *et al.* 1990a). KRDS inhibited synthesis of thromboxane  $A_2$  *ex vivo* in animals and *in vitro* on human and animal platelets (Caen *et al.* 1992). KRDS has been found to inhibit arachidonic acid-induced platelet aggregation and thromboxane formation in dog platelets (Drouet *et al.* 1990a). KRDS, at  $\text{IC}_{50} = 500 \mu\text{M}$ , was a potent inhibitor of ADP-induced rat platelet aggregation *in vitro*, whereas RGDS had no effect ( $\text{IC}_{50} = 10 - 2\text{M}$ ) (Wu *et al.* 1992; Jolles *et al.* 1993). With guinea-pig platelets, both KRDS and RGDS inhibited ADP-induced platelet aggregation with  $\text{IC}_{50} = 600 \mu\text{M}$  and  $\text{IC}_{50} = 150 \mu\text{M}$ , respectively (Wu *et al.* 1992; Jolles *et al.* 1993).

In *in vivo* studies KRDS has been found to be antithrombotic in three different experimental thrombosis models in four different animal species (Caen *et al.* 1992). In dogs, KRDS was an inhibitor of arterial thrombus formation (Wu *et al.* 1992). When tested *in vivo* in rats using an experimental model of laser-induced arterial thrombosis, KRDS was more effective than RGDS in inhibiting thrombus formation at low concentrations, whereas in guinea pigs the reverse was the case (Jolles *et al.* 1993). KRDS and RGDS also acted synergistically in the inhibition of *in vivo* thrombus formation, especially in rats, exerting a highly significant inhibitory effect at individual concentrations which were not effective when administered on their own (Jolles *et al.* 1993). It is, therefore, likely that their mechanisms of action and/or their binding sites are different and specific for the sequence, especially as a similar sequence (KRDG) had little activity. One interesting aspect is, perhaps, the longevity of the antithrombotic effect (80 min) compared with the 100–200 s expected for the small peptides (Bal dit

Sollier *et al.* 1990; Jolles & Caen, 1991; Mazoyer *et al.* 1992; Wu *et al.* 1992).

### Applications

It is not known if peptides with antithrombotic effects are released after milk digestion and absorbed into the bloodstream. But if they do modify human platelet function *in vivo*, then a milk diet could have important antithrombotic properties.

It has been suggested that GMP can be used to treat or prevent thrombosis (Drouet *et al.* 1990b). For intravenous usage the recommended adult dose level is 15–30 mg/kg twice daily for prophylaxis. For oral administration, an adult dose of 50–100 mg/kg twice daily has been recommended (Drouet *et al.* 1990b). Since GMP has no toxic effect, the dose rates can be varied depending on the sensitivity and/or tolerance of the patient.

There has been considerable research activity on the antithrombotic action of the RGDX sequences of fibrinogen  $\alpha$ -chain. However, since the RGDS sequence has been found to induce detachment of endothelial cells *in vitro*, serious concerns exist about general toxicity from the injection of these sequences *in vivo*. KRDS, a related sequence that occurs in human lactoferrin, is not thought to have the potentially detrimental effects of RGDX. KRDS is also antithrombotic, but its mechanism of action and/or its binding site may be different from RGDX. Thus, although it was identified by sequence homology studies between fibrinogen and milk proteins, it has novel antithrombotic activity. All research on this peptide has been based on *in vitro* and/or *in vivo* intravenous treatment in animal models. It is therefore not known if KRDS would be effective when administered orally.

These milk peptides could be considered as possible food additives to either provide treatment of certain conditions or to stimulate the immune system and enhance general health and well-being.

### Conclusions

Milk and milk proteins are well known as excellent nutritional sources. There is a large body of evidence that milk proteins contain or via digestion are the source of a number of biologically active peptides with opioid, immunostimulatory and antihypertensive activities. Several peptides derived from  $\kappa$ -casein and lactoferrin have been shown to be inhibitors of platelet aggregation and to display antithrombotic activity. However, it remains to be determined whether these bioactive peptides are physiologically released and absorbed. The prospect of a natural milk-based diet having antithrombotic properties is very exciting. Alternatively, the possibility remains that these peptides could be generated *in vitro* and used as food additives or molecular models for the design of a new generation of antithrombotic agents.

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