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Binding of Collagen to *Staphylococcus aureus* Cowan 1

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Collagen binds to a receptor protein present on the surfaces of *Staphylococcus aureus* cells. Binding of ^{125}I-labeled type II collagen to its bacterial receptor is reversible, and Scatchard plot analysis indicates the presence of one class of receptor that occurs on an average of 3×10^4 copies per cell and binds type II collagen with a K_d of 10^{-7} M. Studies on the specificity of collagen cell binding indicate that the receptor does not recognize noncollagenous proteins but binds all of the different collagen types tested (types I to VI). Furthermore, isolated collagen α chains and peptides generated by cyanogen bromide cleavage of type I collagen α chains are recognized by the receptor as indicated by the ability of these polypeptides to inhibit binding of ^{125}I-labeled type II collagen to staphylococcal cells. Synthetic collagen analogs were tested as inhibitors of type II collagen binding to bacterial cells. The peptides (Pro-Gly-Pro)$_n$, (Pro-Pro-Gly)$_{10}$, and (Pro-OH-Pro-Gly)$_{10}$ were recognized by the receptor, whereas the peptides (Pro-Ala-Gly)$_n$ and polyproline showed no inhibitory activity.

The matrix proteins fibronectin and laminin may mediate the substrate adhesion of eucaryotic cells, and putative receptors present on the surfaces of cells are believed to recognize distinct sites in the adhesive proteins (35). Some eucaryotic cells (e.g., hepatocytes and chondrocytes) may also adhere to collagen substrate, and the presence of collagen-specific receptors has been implicated (18, 22). The collagen receptor on hepatocytes appears to have broad specificity, since it binds to several different types of collagen as well as to isolated collagen α chains, collagen peptides, and synthetic collagen analogs (22).

In the process of tissue adherence of pathogenic bacteria, structures at the host cell surface or in the extracellular matrix are recognized by specific receptors present on the bacterial cells. Previous studies have shown that bacteria may bind to fibronectin (7, 15, 23, 25, 26, 32), laminin (7, 16, 27, 29, 32), and collagen (3, 7, 12, 32) and that these interactions may in fact represent a mechanism of tissue adhesion. For example, enterotoxigenic *Escherichia coli* only adheres to cultured fibroblasts if the bacteria have a fibronectin receptor, and preincubation of the bacterial cells with soluble fibronectin blocks bacterial adhesion to the cultures (8). Furthermore, fibronectin-binding components have been isolated from cells of *Staphylococcus aureus* (5, 23). These bacteria have been demonstrated to bind to the N-terminal region of fibronectin with high affinity (19).

In this communication we report on the binding of ^{125}I-collagen to a receptor present on *S. aureus*. The collagen receptor interaction is characterized in terms of the structural requirements of the collagen.

(A preliminary account of these findings was presented previously [P. Speziale, M. Höök, and T. Wadström, Proceedings of the V International Symposium on Staphylococci and Staphylococcal Infections, Warsaw, Poland, 1984].)

MATERIALS AND METHODS

Chemicals. Type II collagen was purified from bovine nasal septum as described by Strawich and Nimmi (28). Native collagen types I and III were isolated with neutral salt from fetal bovine skin and purified by NaCl precipitation and DEAE chromatography (30). Type IV collagen was isolated from mouse tumor (31). Type V and VI collagens were purified from a pepsin digest of human placenta (1, 20). The 1(I) and 2(I) chains both from calf and rat skin type I collagens were purified by carboxymethyl cellulose chromatography (21). The rat α chains were treated with cyanogen bromide, and the generated peptides (CB peptides) were purified as previously described (2, 6). The isolated peptides included CB2, CB5, CB6, CB7, and CB8 from the 1(I) chain and CB3, CB4, and CB5 from the α2 chain of rat type I collagen (see Table 2). The synthetic collagen analogs (Pro-OH-Pro-Gly)$_{10}$ and (Pro-Pro-Gly)$_{10}$ were gifts from J. Engel, Basel, Switzerland. Polyproline was from Sigma Chemical Co. (St. Louis, Mo.), and the synthetic (Gly-Ala-Pro)$_n$ and (Pro-Gly-Pro)$_n$ were from Miles Scientific (Div. Miles Laboratories, Inc., Naperville, Ill.). Na^{125}I (specific activity, 15 mCi/μg) was from Amersham Corp. (Arlington Heights, Ill.).

Type II collagen was labeled with ^{125}I by the chloramine T method (13). The specific activity of the radioactively labeled protein was estimated to be 1.8×10^6 cpm/μg. Fibronectin was purified from human plasmid as previously described (33). Human fibrinogen was a gift from G. de Petro, Brescia, Italy. Bovine serum albumin, bovine immunoglobulin G, α_1 acid glycoprotein, trypsin, soy bean trypsin inhibitor (type 1-S), papain, protein A, sodium β-glycerophosphate, and gelatin were from Sigma.

Bacteria. *S. aureus* Cowan 1, obtained from the Instituto Seroterapico Milanese (Milan, Italy), was cultured under constant rotation for 15 h at 37°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) supplemented with 5 g of sodium β-glycerophosphate, 5 g of D-glucose, and 6.5 g of yeast extract per liter. Bacteria were harvested by centrifu-

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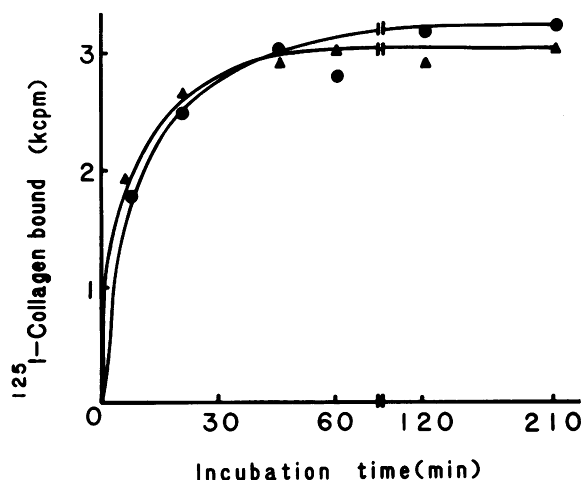

FIG. 1. Time course of binding of ^{125}I-collagen to cells of *S. aureus* Cowan 1. Staphylococcal cells were incubated before (▲) and after (●) heat treatment with ^{125}I-collagen for the indicated periods, and binding was assayed as described in Materials and Methods. The data refer to the amount of protein bound to 10^7 cells.

gation (8,000 × *g*, 20 min), suspended, and washed twice in phosphate-buffered saline (PBS, pH 7.4, containing 0.13 M sodium chloride, 10 mM phosphate buffer, and 0.02% sodium azide to suppress bacterial growth). Cells were then suspended in PBS and heated at 88°C for 15 min to kill bacteria and inactivate hydrolases. The cell suspension was stored at −70°C until used. The number of cells in a suspension was determined spectrophotometrically with a previously prepared standard curve relating A_{600} to the cell number determined by counting cells in a Petroff-Hausser counting chamber.

Binding assay. Staphylococcal cells (5×10^7) were incubated in a total volume of 0.6 ml with 5×10^4 cpm of ^{125}I-collagen type II in PBS containing 0.1% bovine serum albumin and 0.1% Tween 80 to minimize nonspecific binding to cells and tubes. The tubes containing the reaction mixtures were incubated end over end at 20°C for 1 h unless otherwise stated. The reaction was stopped by addition of 3 ml of ice-cold 0.1% Tween 80 in PBS, and the tubes were centrifuged at 1,350 × *g* for 20 min. After aspiration of the supernatant, the tubes, which contained the bacterial pellet, were analyzed for radioactivity in a gamma counter (LKB Wallac, Turku, Finland). Duplicate samples were analyzed, and background values representing radioactivity recovered in the tubes incubated in the absence of bacteria were subtracted. This value usually did not exceed 1% of the total radioactivity added.

Incubation of bacteria with proteolytic enzymes. Staphylococcal cells (200 mg, wet weight) were suspended in 1 ml of PBS (without azide) and digested with trypsin (25 μg/ml) or papain (10 U/ml) at 37°C. At the indicated times, samples were removed from the incubation mixture, and soybean trypsin inhibitor (50 μg/ml) was added to the trypsin-digested samples. All samples were heated for 10 min at 88°C. Cells were pelleted by centrifugation, washed, suspended in PBS, and assayed for collagen-binding activity.

RESULTS

Initial screening of *S. aureus* strains for ability to bind ^{125}I-collagen showed that 12 of 37 strains bound the labeled proteins at a level of at least 2% (1,000 cpm) of the total radioactivity added. These strains were considered to be collagen binders. Within this group of strains the amount of bound ^{125}I-collagen varied, reaching 40% for some strains. One of the strains binding the highest amount of ^{125}I-labeled type II collagen (*S. aureus* Cowan 1) was selected for further study.

Binding of ^{125}I-labeled type II collagen to *S. aureus*. The time course of binding of ^{125}I-labeled type II collagen to live staphylococcal cells is shown in Fig. 1. Under the conditions used, the reaction was rapid, and after 20 min of incubation essentially maximal binding of ligand to staphylococcal cells was observed.

Heat killing the bacterial cells (88°C for 15 min) did not change the course of time-dependent binding of ligand nor the amount bound to bacterial cells (Fig. 1). Heat-killed bacteria were used throughout this investigation, and bacteria were routinely incubated with ligand for 60 min.

A major portion of the ^{125}I-collagen bound to the bacteria could be displaced from the cells by addition of unlabeled collagen, demonstrating reversibility of the collagen-bacteria interaction. The time course of this reaction is shown in Fig. 2. Bacterial cells were first incubated with ^{125}I-collagen for 1 h. Subsequently, 100 μg of unlabeled collagen was added, which resulted in rapid displacement of the majority of radiolabeled ligand from the cells.

Incubation of bacterial cells with increasing amounts of collagen resulted in increasing binding of ^{125}I-labeled ligand up to a level at which the cells had apparently been saturated with ligand (Fig. 3). This indicates the presence of a limited number of collagen-binding sites on the bacterial cells. Since 5×10^7 staphylococcal cells can bind a maximum of 0.8 μg of type II collagen ($M_r = 2.85 \times 10^5$; 28), this corresponds to an average of 3×10^4 collagen-binding sites per cell. Scatchard plot analysis (24) of the binding data fitted a straight line (Fig. 3, insert), indicating the presence of one class of collagen receptor. From the slope of the line a dissociation constant of 10^{-7} M could be calculated for the *S. aureus* Cowan 1 receptor-collagen type II interaction.

Specificity of collagen binding to *S. aureus*. To analyze the specificity of collagen binding to staphylococci, we incu-

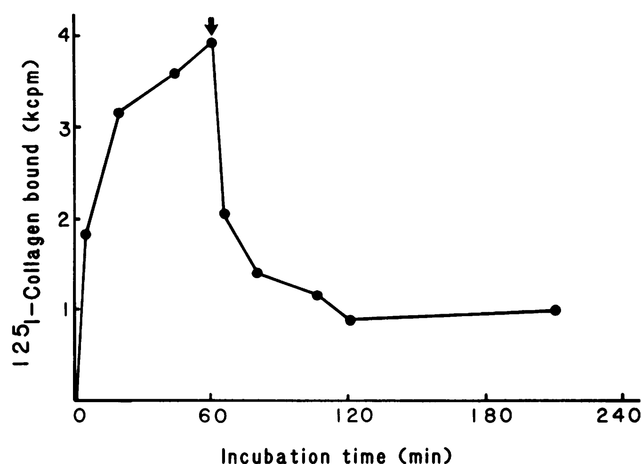

FIG. 2. Reversibility of binding of ^{125}I-collagen to *S. aureus* Cowan 1. Staphylococci were incubated with 25 ng of ^{125}I-collagen for 1 h. Following this, as indicated by the arrow, the incubation mixture was supplemented with 100 μg of unlabeled collagen. The amount of bound ^{125}I-collagen was assayed as described in Materials and Methods.

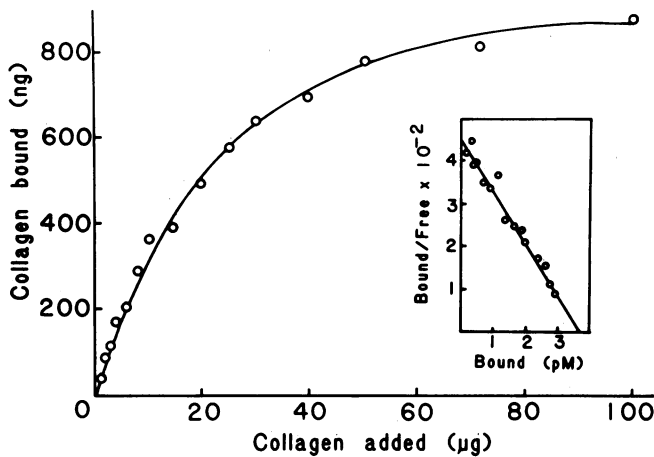

FIG. 3. Saturability of binding of ^{125}I-collagen to *S. aureus* Cowan 1. Bacteria were incubated with increasing amounts of ^{125}I-labeled collagen (specific activity, 2,350 cpm/μg). Data refer to the amount of collagen bound by 5×10^7 cells. Background values were determined for each concentration of added ^{125}I-collagen and subtracted. Inset: Scatchard plot analysis of the above data.

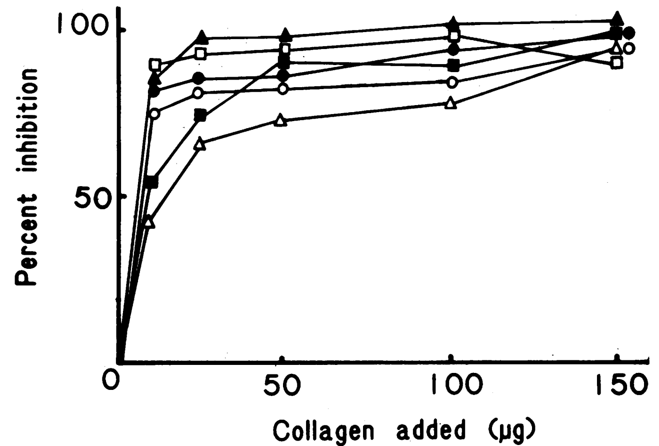

FIG. 4. Effects of different collagen types on ^{125}I-collagen binding to *S. aureus* Cowan 1. Collagens of types I (○), II (▲), III (●), IV (△), V (■), and VI (□) were added to incubation mixtures containing ^{125}I-collagen type II and bacteria and incubated for 1 h, and the amount of bound ^{125}I-collagen was assayed. Inhibition is expressed as percent ^{125}I-collagen bound to bacteria in the absence of any potential inhibitor.

bated cells with ^{125}I-labeled type II collagen in the presence of 200 μg of unlabeled inhibitors. As expected, unlabeled type II collagen was an effective inhibitor of the binding of ^{125}I-collagen to cells (Table 1). Of the other proteins tested, orosomucoid, immunoglobulin G, ovalbumin, fibronectin, and staphylococcal protein A did not affect the binding of ^{125}I-collagen to bacterial cells (Table 1), indicating the presence of receptors specific for collagen. Fibrinogen had some inhibitory activity. The *S. aureus* Cowan 1 used in this study also binds fibronectin, fibrinogen, and immunoglobulin G, and it is possible that the collagen and fibrinogen receptors are located at close proximity and that receptor-bound fibrinogen, for steric reasons, interferes with the binding of collagen to its receptor.

When different types of collagen (I to VI) were included in the incubation mixture, they all inhibited the binding of ^{125}I-collagen type II to bacterial cells (Fig. 4). Minor differences in inhibitory activity were observed among the different collagen types. Type IV collagen appeared to have the lowest inhibitory activity. However, it should be noted that 150 μg of this protein inhibited cell binding of ^{125}I-collagen type II by 90%, whereas noncollagenous proteins had essentially no inhibitory activity. Isolated collagen type I α chains obtained from rat and calf collagen type I (Table 1), as well as denatured collagen (data not shown), exhibited strong inhibitory activities (Table 1). These observations suggest an absence of species specificity in collagen binding to staphylococcal cells.

Different collagen peptides isolated after degradation of type 1 collagen with CNBr all inhibited binding of type II collagen by bacterial cells, although the extent of inhibition varied. Whereas α2(I) CB4, α1(I) CB6, and α1(I) CB7 inhibited the cell binding of ^{125}I-collagen to more than 90%, the same amount of α1(I) CB2 and α1(I) CB5 only caused 50% inhibition. This difference in inhibitory activities may be a reflection of size differences. The peptides with good inhibitory activity were generally larger than those with poor inhibitory activity (Table 2).

The observation that all collagen types and CB peptides inhibited binding of ^{125}I-labeled collagen type II to bacterial cells suggests that the collagen receptor on *S. aureus* Cowan I recognizes a structure common to all collagens. To examine the specificity of the collagen receptor further, synthetic peptides with structures analogous to those of the collagens were tested as potential inhibitors. The results of these

TABLE 1. Specificity of binding of ^{125}I-collagen to *S. aureus* Cowan 1[a]

Protein	% Inhibition
Collagen type II	89
Fibronectin	−2
Fibrinogen	12
Immunoglobulin G	0
Egg albumin	1
α1-acid glycoprotein	0
Protein A	−1

[a] Bacterial cells (5×10^7) were incubated in the presence of 5×10^4 cpm of ^{125}I-collagen (25 ng) and 100 μg of competing proteins. The amount of radioactivity recovered in the tubes in the absence of proteins was set as 0% inhibition, and all data are expressed as a percentage of the control.

TABLE 2. Effect of collagen chains and peptides on ^{125}I-collagen binding to *S. aureus* Cowan 1[a]

Peptide	Size of peptide (no. of amino acid residues)	% Inhibition
α1(I) chain (rat)	1,050	84
α1(I) chain (calf)	1,050	90
α2(I) chain (rat)	1,050	90
α2(I) chain (rat)	1,050	90
α1(I) CB2	36	49
α1(I) CB5	37	52
α1(I) CB6	196	100
α1(I) CB7	271	90
α1(I) CB8	279	88
α2(I) CB3	321	79
α2(I) CB4	335	91
α2(I) CB5	325	87

[a] Two hundred micrograms of α-chain or cyanogen bromide (CB) peptide was added to incubation mixtures at the same time as radiolabeled collagen. Binding is expressed relative to binding by bacteria incubated in the absence of any potential inhibitor.

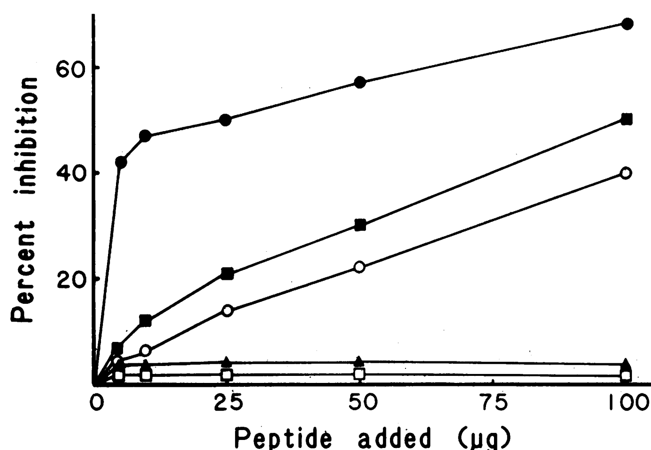

FIG. 5. Effect of synthetic peptides on ^{125}I-collagen binding to *S. aureus* Cowan 1. Bacteria were incubated in the presence of polyproline (▲), (Pro-Pro-Gly)$_{10}$ (○), (Pro-OH-Pro-Gly)$_{10}$ (■), (Pro-Gly-Pro)$_n$ (●), or (Gly-Ala-Pro)$_n$ (□). Data are expressed as a percentage of inhibition of the control, i.e., incubation performed in the absence of unlabeled peptides.

experiments (Fig. 5) showed that the peptide (Pro-Gly-Pro)$_n$ was the most efficient inhibitor. The binding of ^{125}I-labeled collagen type II to staphylococcal cells was inhibited to 50% by 20 μg of the peptide. The peptides (Pro-OH-Pro-Gly)$_{10}$ and (Pro-Pro-Gly)$_{10}$ also exhibited inhibitory activity, although more than 100 μg of each peptide was required to achieve 50% inhibition. Polyproline and (Gly-Ala-Pro)$_n$ showed no inhibitory activity.

Nature of the collagen receptor. Previous studies of bacterial receptors for connective tissue proteins have indicated that these receptors are mostly proteins. If the staphylococcal collagen receptor were also a protein, one would expect the collagen-binding potential of a bacterium to be reduced after digestion of the cell with proteases. Figure 6 shows the amounts of ^{125}I-collagen bound to *S. aureus* Cowan 1 after incubation of cells for increasing periods with papain or trypsin. Both treatments eventually resulted in total loss of collagen binding, suggesting that the collagen receptor contained a protein component.

DISCUSSION

The existence of collagen-binding proteins has been reported by Chiang and Kang (4) in platelet membranes. Others have also described an integral membrane protein responsible for direct interaction of the chondrocyte surface with type II collagen (17, 18). Specific binding to collagen has been shown for both fibroblasts (9, 10) and hepatocytes (22).

In the present communication, we report that some strains of *S. aureus* recognize and bind collagen. Binding of ^{125}I-collagen to bacteria is rapid and reversible and involves a limited number of bacterial binding sites, corresponding to 3 × 10^4 receptor sites per cell. Scatchard plot analysis (22) of the binding data indicates the presence of one class of collagen receptor which binds the ligand with a K_d of 10^{-7} M. Binding of ^{125}I-collagen to bacteria exhibits a high degree of specificity in that the presence of unlabeled collagen, but not that of unrelated proteins, inhibits binding. Fibrinogen is an exception, as its presence causes a small but significant reduction of ^{125}I-collagen binding. However, it is unlikely that fibrinogen and collagen bind to the same receptor site, since strains of staphylococci (e.g., *S. aureus* Newman) exist that bind fibrinogen but not collagen (data not shown). It is possible that fibrinogen and collagen receptors are located in close proximity on the bacterial surface and that fibrinogen bound to its receptor for steric reasons interferes with the interaction between the collagen receptor and its ligand.

The ligand specificity of the collagen receptor on *S. aureus* is similar to that previously described for a collagen receptor on rat hepatocytes (22). Both receptors recognized all collagen types tested (i.e., types I to VI) and also all peptides generated by CNBr cleavage of type I or III collagen, and a higher apparent affinity was observed for larger CNBr peptides than smaller ones. Furthermore, both receptors appear to recognize synthetic peptides with structures which contain repeating triplets similar to those found in collagen. However, an apparent difference in the substrate specificity of the two collagen receptors is reflected by the observation that the hepatocyte, but not the bacterial receptor, recognizes the peptide structure Gly-Ala-Pro, which is also a typical collagen structure.

With the specific binding of collagen to *S. aureus* reported in this communication, collagen is added to the list of connective tissue proteins specifically recognized by pathogenic bacteria. Treatment of bacteria with pepsin or trypsin resulted in loss of collagen binding, suggesting that the collagen receptor has a protein component. However, the collagen receptor differs from other receptors of matrix proteins in that it has considerably lower apparent affinity for its ligand compared with those reported for fibrinogen (11, 14), fibronectin (23), and laminin (15), which bind their respective ligands with K_ds on the order of 10^{-9} M. On the other hand, the collagen receptor appears to be a more abundant protein on bacterial cells than previously reported receptor proteins. Considering that, in tissue, collagen occurs in multimolecular fibrils, it appears likely that several receptor molecules on a bacterial cell could bind to the same fibril, resulting in strong binding between the bacterial cell and collagen fibrils. Hence, the binding of *S. aureus* to collagen may represent a mechanism of tissue adherence that is of particular importance in bacterial colonization of

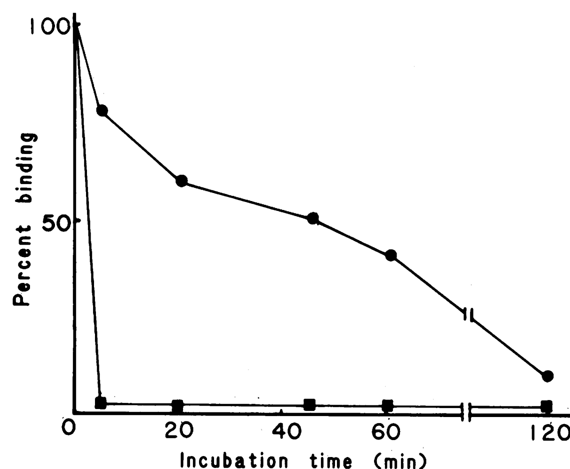

FIG. 6. Binding of ^{125}I-collagen to trypsin- or papain-digested *S. aureus* Cowan 1. Bacteria were preincubated with papain (10 U/ml) (■) or trypsin (25 μg/ml) (●), harvested, and then assayed for ^{125}I-collagen binding. Binding is expressed as a percentage of binding to an untreated control.

tissues like bone or cartilage, where staphylococcal infections commonly occur (34).

ACKNOWLEDGMENTS

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