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# PARTIAL PURIFICATION AND SPECIFICITY OF AN ARGININE-CONVERTING ENZYME FROM BOVINE EPIDERMIS

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## Summary

An enzyme which catalyzes the conversion of intraprotein arginine residue to intraprotein citrulline residue is present in bovine snout epidermis. This arginine-converting enzyme has been partially purified by $(NH_4)_2SO_4$ precipitation chromatography on DEAE-cellulose and chromatography on Sephadex G-200. The enzyme is active at neutral pH, requires $Ca^{2+}$ and a reducing agent and has an apparent molecular weight of 69 000. Its substrates include histone, polyarginine, *S*-carboxymethyl cysteine-hair keratin, *S*-carboxymethyl cysteine epidermal keratin and prekeratin and *S*-carboxymethyl cysteine-trichohyalin. A large number of proteins, synthetic and naturally occurring peptides, and other guanidine-containing compounds were not substrates of the arginine-converting enzyme.

## Introduction

The presence of citrulline in the proteins of hair follicle and inner root sheath has been unequivocally established by Rogers and co-workers [1—4]. This amino acid is formed by the action of an arginine-converting enzyme on arginine residues of specific hair follicle proteins [5,6]. In recent reports citrulline was shown to be present in stratum corneum proteins of mammalian epidermis [7,8]. In addition we demonstrated an epidermal enzyme which, like hair follicle arginine-converting enzyme, catalyzes the arginine to citrulline conversion in the hair follicle protein trichohyalin [9].

Abbreviations: BME, β-mercaptoethanol; HRP, histidine-rich protein.

The present work describes the partial purification and properties of the epidermal enzyme and the demonstration of natural substrates within the epidermis.

## Materials and Methods

*Crude enzyme.* Black snouts from 10-week-old calves were purchased from a local packing house. Epidermal slices were homogenized in a VirTis homogenizer at 1 g of tissue per 10 ml of 5 mM Tris-HCl/2 mM EDTA (pH 7.1). After stirring for 1 h, the homogenate was centrifuged at 10 000 × *g*. The clear supernate contained the arginine-converting activity and was designated the crude enzyme extract.

*Enzyme assay.* Incubation of crude extract with either trichohyalin or prekeratin produced measurable citrulline after 20 h, but the reaction rate was not linear with time. Attempts to measure initial rates were not precise, because citrulline levels in the first hours were insufficient for accurate measurement. Purification was, therefore, monitored by incubating enzyme-containing samples with prekeratin for 24 h, followed by citrulline analysis. Samples which contained the enzyme were readily identified, but the amount of activity could not be accurately quantitated. Specifically, 2.0 ml of reaction mixture contained 0.5 ml of enzyme solution, 1.0 mg of bovine *S*-carboxymethyl cysteine-prekeratin and was 10 mM in Tris-HCl, pH 7.1, 10 mM in cysteine, 10 mM in $CaCl_2$ and 50 μg/ml in gentamycin. After 24 h incubated samples were dialyzed in water, hydrolyzed in 6 N HCl and citrulline contents were determined as described previously [7].

*Partially purified enzyme.* Crude enzyme extract was dialyzed against 5 mM Tris-HCl/2 mM EDTA (pH 7.1). Solid $(NH_4)_2SO_4$ was added to 70% saturation and the precipitated protein was redissolved in 5 mM Tris-HCl/2 mM EDTA/4 mM NaCl (pH 7.1). After dialysis against the same buffer, the sample was applied to a 2.5 × 90 cm DEAE-cellulose column and eluted by increasing the NaCl to 50 mM. Active fractions were dialyzed vs. 5 mM Tris-HCl/2 mM EDTA/4 mM NaCl (pH 7.1) and applied to a second DEAE-cellulose column equilibrated in the same buffer. The enzyme was eluted with a 4—50 mM NaCl gradient. Active fractions were combined and concentrated by ultrafiltration against an Amicon PM-30 membrane to a volume of 5—10 ml. The concentrated preparation was dialyzed into 5 mM Tris-HCl/2 mM EDTA/100 mM NaCl (pH 7.1) and chromatographed on a 1.5 × 90 cm column of Sephadex G-100. The combined active fractions constituted the partially purified enzyme.

*Preparation of protein substrates.* Prekeratin and keratins soluble in 8 M urea/0.1 M β-mercaptoethanol/0.1 M Tris-HCl (pH 9.0) (urea-BME proteins) were prepared as previously described [7]. Keratohyalin was isolated from bovine hoof [10] and $HRP_1$ and $HRP_2$ from newborn rat epidermis [11] were provided by Dr. Isadore Bernstein. Human hair keratin was prepared as previously described [12]. (*Guanido*-$^{14}$C)-labelled trichohyalin was prepared from guinea-pig hair follicle as follows: 50 μCi of (*guanido*-$^{14}$C)-labelled L-arginine (40 mCi/μmol) was injected intraperitoneally into 200-g guinea-pigs, the trunks of which had been epilated by the wax-sheet method 7 days previously. After

4 h animals were re-epilated, and the hair follicle-containing wax sheets were chilled to −60°C and pulverized. Wax was dissolved by stirring in chloroform at room temperature for 2 h and the hair bulbs were collected on a Buchner funnel. Extraction and purification of trichohyalin from the hair bulbs was done as described by Rogers et al. [6]. Cultured human cell prekeratin and urea-BME proteins were labelled and prepared as previously described [13]. Calf thymus histone was purchased from Vega Biochemicals, Tucson, AZ. Polyarginine sulfate (average $M_r$ 70 000) was purchased from Sigma Chemical Co., St. Louis, MO and polyarginine chloride (average $M_r$ 15 000) was purchased from Miles Laboratories, Elkhart, IN. All epidermal proteins were converted to their *S*-carboxymethyl cysteine (SCM) derivatives as previously described [14]. All peptides and guanidine groups containing compounds were obtained commercially, and were the best grades available.

*Identification of substrates.* 20 μg of partially purified enzyme catalyzed maximum conversion in 1 mg of bovine prekeratin in 20 h. To determine maximum conversion in other potential unlabelled substrates, 1 mg was incubated for 24 h with 50 μg of partially purified enzyme under the same conditions used to monitor the enzyme purification.

For citrulline production in (*guanido*-$^{14}$C)-labelled arginine substrates 1000 cpm of protein (0.30 mg trichohyalin, 10 μg human cell prekeratin, or 25 μg human cell urea-BME proteins) were incubated. Citrulline was separated from the hydrolyzed reaction mixture with an amino acid analyzer and quantitated by the ninhydrin reaction or by scintillation counting [7]. Assays containing dialyzable substrates were also incubated for 24 h at 37°C but were terminated by the addition of 70% $HClO_4$ to a concentrationof 5%. After centrifugation these assays were neutralized to pH 7.0 with KOH and centrifuged. Clear supernatants solutions were lyophilized and hydrolyzed for 24 h in 6 N HCl. All hydrolyzed samples were evaporated to dryness and redissolved in water. Citrulline was determined by reaction with the diacetylmonoxime reagent [15].

## Results

The crude extract was precipitated with $(NH_4)_2SO_4$, redissolved, dialyzed and chromatographed on DEAE-cellulose in the presence of 4 mM NaCl. After washing with starting buffer, activity was eluted with 50 mM NaCl in a concentrated peak. When the 50 mM peak was rechromatographed on DEAE-cellulose, a 4—50 mM linear NaCl gradient consistently eluted the activity at 15—20 mM (Fig. 1). After an ultrafiltration step the preparation was chromatographed on Sephadex G-100 (Fig. 2). The molecular weight of the partially purified enzyme on Sephadex G-100 was determined to be 69 000. Chromatography of the same preparation or of crude enzyme on Sephadex G-200 gave the same molecular weight.

Crude extract contained 1400 mg of protein, of which at least 1.5 mg was necessary to obtain maximum conversion in *S*-carboxymethyl cysteine-prekeratin in 24 h. Partially purified enzyme contained on the average 2 mg of protein of which 0.020 mg was necessary for maximum conversion of prekeratin in 24 h. Thus, the fractionation steps had removed over 99% of the pro-

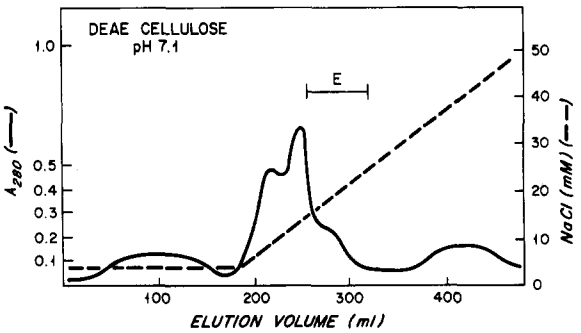


Fig. 1. Chromatography of the enzyme on DEAE-cellulose. The dialyzed 50 mM peak from column 1 in 5 mM Tris-HCl/2 mM EDTA/4 mM NaCl, pH 7.1, was applied to a 1.5 × 60 cm at 14 ml/h. After washing with starting buffer, the enzyme (E) was eluted in a 4—50 mM linear NaCl gradient over 24 h.

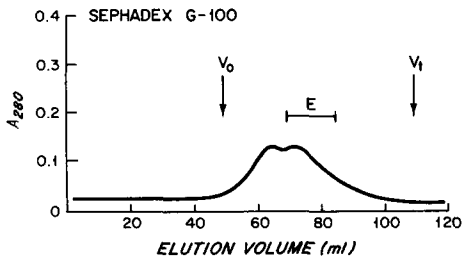


Fig. 2. Chromatography on Sephadex G-100. Concentrated active material from DEAE-cellulose column (5—10 ml) in 5 mM Tris-HCl/2 mM EDTA/100 mM NaCl was chromatographed on a 1.5 × 90 cm column at 10 ml/h.

TABLE I

LIST OF PROTEINS, PEPTIDES AND GUANIDINE GROUP CONTAINING COMPOUNDS WHICH WERE NOT SUBSTRATES

1 mg was incubated with 50 μg of partially purified enzyme for 24 h at 37°C in the presence of $CaCl_2$ and cysteine. Citrulline could not be detected in the hydrolyzed assay mixture.

| | |
|---|---|
| Arg | Leu Trp Met Arg Phe Ala |
| Agr Glu | Benzoyl arginine ethyl ester |
| Arg Val | Benzoyl arginine amide |
| Arg Pro | Benzoyl arginine-*p*-nitroanilide |
| Arg Tyr | α-Amino-guanido butyrate |
| Arg Ala | *p*-Guanidino benzoate |
| Arg Leu | Arginine phosphate |
| Arg Gly | Streptomycin |
| Arg Asp | Bradykinin |
| Arg Phe | Keratohyalin |
| Val Arg | Protamine |
| Met Arg Phe | Aprotinin |
| Arg Phe Ala | Casein |
| Leu Trp Met Arg | Glucagon |
| Arg Pro Pro Arg | $HRP_1$ |
| Met Arg Phe Ala | $HRP_2$ |
| Leu Trp Met Arg Phe | |

TABLE II

SUBSTRATES OF BOVINE EPIDERMAL ARGININE-CONVERTING ENZYME

1 mg of unlabelled or 1000 cpm of labelled protein was incubated with 50 μg of purified enzyme at 37°C. After 24 h, citrulline was determined in the assay mixtures.

| Protein * | Citrulline (nmol/mg) | Arg (%) |
|---|---|---|
| Prekeratin (bovine) | 80 | 20 |
| Urea-BME keratin (bovine) | 50 | 14 |
| Hair keratin (human) | 40 | 7 |
| Prekeratin (human culture) | 140 | 25 |
| Urea-BME (human culture) | 80 | 14 |
| Trichohyalin (guinea-pig) | 100 | 18 |
| Histone (bovine) | 25 | 3 |
| Polyarginine ($M_r$ 70 000) | 30 | 0.05 |
| Polyarginine ($M_r$ 15 000) | 3—5 | 0.008 |

* Keratins and trichohyalin were *S*-carboxymethyl cysteine derivatives.

tein, yet retained about 10% of the activity indicating that significant purification had been achieved.

Table I is a list of all compounds in which citrulline was not produced when incubated with the enzyme, while in Table II both the amount of citrulline produced and the percent of arginine residues converted are shown for those substrances found to be substrates. It should be noted that substrates and enzymes were incubated singly or together in assay buffer. In no case was citrulline produced in the absence of enzyme, however some enzyme preparations contained low levels of endogenous peptide-bond citrulline which was increased by incubation.

## Discussion

Arginine to citrulline converting activity has been observed in guinea-pig hair bulb [5,6] and in bovine snout epidermis [9]. The partially purified epidermal enzyme is active against several proteins including many fibrous proteins. With these proteins, it has proven difficult to develop an enzyme assay which is reliable, as the conversion rates are not linear with respect to time. A convenient but quantitative assay would require a substrate containing a high concentration of convertible arginine residue in which citrulline production would be proportional to time and be measurable by chemical means without requiring hydrolysis and separation on an amino acid analyzer. To find such a substrate a large number of commercially available peptides were incubated with partially purified enzyme, but in none of these was a measurable amount of citrulline produced in a 24 h period.

Proteins of bovine snout epidermis were also examined and both prekeratin and the urea-BME-soluble proteins were excellent substrates as were the keratin and prekeratin of cultured human keratinocytes. Keratinocyte keratins are similar to tissue keratins in immunological reactivity and amino acid content but differ in their SDS-polyacrylamide gel patterns [13,16]. Additional substrates include *S*-carboxymethyl cysteine hair keratin, calf thymus histone and polyarginine. That the polyarginines are substrates indicates that convertible

arginines are found in uninterrupted arginine sequences; however the convertibility of the hair, epidermal, and keratinocyte keratins, which have arginine contents of only 4—6 residues/100 residues, suggests that convertible arginines lie in other sequences as well.

It is difficult to assess the biological significance of the arginine-converting enzyme. In hair follicle large amounts of protein-bound arginine are modified to protein-bound citrulline. The mechanism by which this is thought to occur is through the converting enzyme which is apparently localized in the medulla and inner root sheath [5]. The process itself appears to be related to hair maturation.

In epidermis only low levels of protein-bound arginine are converted to protein-bound citrulline presumably by the epidermal enzyme.

Prekeratin and stratum corneum fibrous protein are good substrates of the epidermal enzyme, yet only stratum corneum fibrous protein contains citrulline [7]. The presence of insoluble citrulline-containing proteins in the stratum corneum is consistent with the barrier function of this tissue in that, these proteins would be less susceptible to any trypsin-like enzymes produced by microorganisms of the skin.

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