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Note

Affinity chromatography of kid chymosin on histidyl-Sepharose

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The cheese industry uses an enourmous quantity of milk coagulating enzyme. Following the scarcity of calf rennin, much work has recently been carried out on renin from the lamb, kid and also that of microbial origin. Although the milk clotting enzymes from the lamb and kid remain largely uninvestigated, certain industries do utilize them for the manufacture of cheese[1]. Since there is little information on chymosin from the kid, this study was undertaken to evolve a method of purification of the enzyme and to characterize it. We describe a chromatographic method based principally on the multiple interactions of histidine with chymosin from the kid.

EXPERIMENTAL

Abomasum from the kid from Granday, France. Sepharose 4B was from pharmacia and L-histidine from Sigma. All other chemicals and reagents were supplied by Merck.

Extraction of the enzyme

The extraction of chymosin was carried out according to a previous method[2] modified as follows. Abomasum fragments were first macerated in a solution of NaCl (6%) and H_3BO_3 (2%). The crude extract obtained was then dialysed against 0.01 *M* sodium acetate, pH 5.5 during 24 h with three changes of the buffer.

Determination of clotting activity

A reconstructed milk from spray-dried milk powder (12%, w/v with 0.01 *M* Ca^{2+}) was used. The coagulation time was measured at 37°C. The enzyme concentration was adjusted so that clotting was visible on the test-tube walls between 1 and 3 min. Clotting activity is calculated as

$$SU = (2400/T) \times 5/0.5 \times D$$

where SU = Sohxlet unit, T = time taken to coagulate and D = dilution factor.

Estimation of proteolytic activity on caesein

The proteolytic activity of chymosin from the kid with respect to casein was

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determined by measuring the increase in the non-proteinaceous nitrogen content in the reaction mixture which is soluble in 0.4 *M* trichloroacetic acid (TCA). The by-products of the proteolysis are also generally soluble in TCA. Following the described procedure[3], modified by using 1 ml of the enzyme solution (diluted 100 times), 1 ml of casein (20%) was added in a water-bath at 40°C. After 10 min, 2 ml of 0.4 *M* TCA were added to the reaction mixture. The precipitate was filtered off through a Millipore filter. To 1 ml of the filtrate, 5 ml of 4 *M* Na_2CO_3 and 1 ml of Folins–Ciocalteau reagent were added. The mixture was then incubated for 20 min at 40°C. Proteolysis is quantified by the increase in tyrosine liberated as determined by the absorbance at 660 nm.

Chromatography

Histidine was coupled to Sepharose 4B as described[4]. This method is based on an epichlorohydrin activation of the matrix in an alkaline medium which results in free oxiran groups. The ligand, histidine, is then immobilized on Sepharose 4B via the oxiran groups. A column of histidyl-Sepharose 4B (20.5 × 1 cm) was equilibrated with 0.1 *M* sodium acetate buffer pH 5.5. The crude extract of the kid corresponding to 11.1 mg of protein was injected and the column washed with the equilibrating buffer. Chymosin was specifically eluted with 0.5 *M* NaCl in the starting buffer.

Histidyl-Sepharose 4B was found to be stable and the column could easily be regenerated. The same column was used at least twenty times with no change in the elution profile.

Characterization of the purified enzyme

The homogeneity of the purified chymosin was checked by sodium dodecyl sulphate (SDS) gel electrophoresis in 10% polyacrylamide gel at pH 7.0. Isoelectric focusing was carried out in the range pH 3.5–9.5 on 0.2-mm thick polyacrylamide gels (LKB). Staining and destaining were carried out according to the LKB instruction (Application Note 306). Gel filtration, on a column of sephacryl S-200 (28.5 × 1 cm) was carried out at room temperature. The column was washed with 50 m*M* sodium acetate buffer, pH 5.5.

RESULTS AND DISCUSSION

Chromatography on histidyl-Sepharose

Amino acids like tryptophan and lysine have been coupled to Sepharose for the purification of proteins[5,6]. Histidyl-Sepharose has been shown to exhibit hydrophobic, ionic and charge-transfer type interactions[4,7].

Tables I and II show the results of purification of chymosin from kid abomasum on this gel. In the case of the crude dialysed extract, an 18-fold purification with a yield of 108% is obtained at 20°C (Fig. 1). Lowering of the elution temperature from 20 to 4°C increases the purification from 18- to 26-fold, and also results in an increase in the activity from 108 to 331% (Fig. 2). This means the chymosine is more specifically adsorbed to the histidine column at lower temperatures. As already discussed[5], at lower temperatures the charge-transfer type of interactions are predominant with amino acid-coupled Sepharose gels, in aqueous media. The presence of histidine and tyrosine residues at the active sites favours such interactions.

TABLE I

PURIFICATION OF CHYMOSIN FROM THE KID ON HISTIDYL-SEPHAROSE 4B AT 20°C

Purification step	*Activity (SU)*	*Protein (mg)*	*Specific activity (U/mg)*	*Activity recovered (%)*	*Protein recovered (%)*	*n-Fold purification*
Crude extract	21,600	27.3	797	100	100	0
Dialysed extract	21,600	9.1	2398	100	33	3
Chromatography at 20°C						
First peak eluted with equilibrating buffer	0	7	0	0	25	0
Second peak eluted with 0.5 *M* NaCl	23,466	1.5	14,777	108	5	18.0

TABLE II

PURIFICATION OF CHYMOSIN FROM THE KID ON HISTIDYL-SEPHAROSE 4B AT 4°C

Purification step	*Activity (SU)*	*Protein (mg)*	*Specific activity (U/mg)*	*Activity recovered (%)*	*Protein recovered (%)*	*n-Fold purification*
Crude extract	20,000	31.0	645	100	100	0
Dialysed extract	20,000	10.9	1834	100	35	2.8
Cromatography at 4°C						
First peak eluted with equilibrating buffer	0	6.4	0	0	20.6	0
Second peak eluted with 0.5 *M* NaCl	66,000	3.9	16,923	331	12.5	26.2

The usual ligand used in affinity chromatography for chymosin purification is pepstatin, a peptide with a statine residue as the essential binding site. Kunimoto *et al.*[8] suggested a hydrophobic mechanism of binding of acid proteases to pepstation. Considering the tetrahydral configuration proposed for the statine residue, the two methyl groups in the residue can contribute to hydrophobic and donor effects. Histidine can participate in similar interactions with the enzyme. However, hydrogen-bond formation between the enzyme and histidine is not to be excluded, as is the case with serine proteases binding to their substrates at histidine[10]. The binding of chymosin to the histidine ligand is specific in solution as shown below.

Inhibition studies with histidine

As shown in Fig. 3, histidine seems to be a good inhibitor with a constant of inhibition (K_i) value of $14.7 \cdot 10^{-3}$ *M* and a concentration of $18.5 \cdot 10^{-3}$ *M* is needed for 50% inactivation of the enzyme. In the case of pepstatine a value of $6 \cdot 10^{-6}$ *M* was reported for 50% inactivation of chymosin[9]. The different statyl derivatives studied by these authors had $K_i$values ranging from $1.4 \cdot 10^{-4}$ to $1.1 \cdot 10^{-9}$. Although histidine seems to be a much less potent inhibitor from these studies, this ligand gives a better purification, compared to the very low yields reported in the case of affinity chromatography with pepstatin Sepharose, *i.e.*, a 26-fold purification with 331%

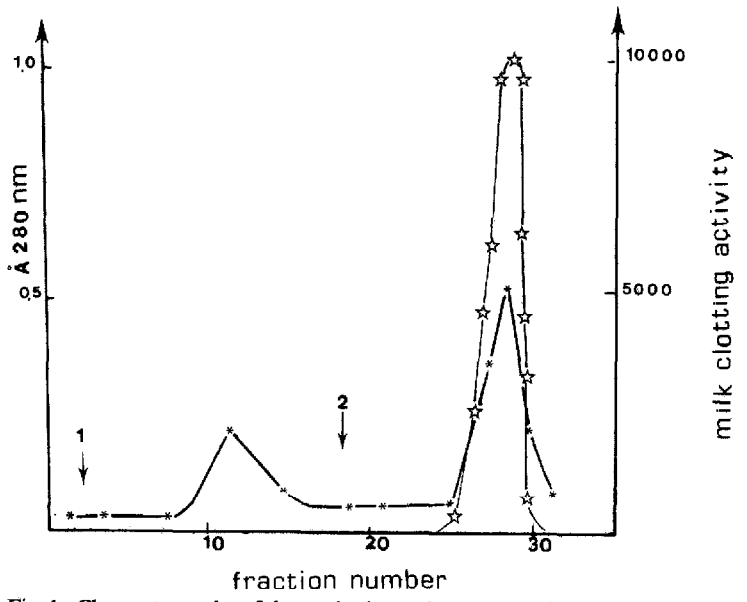


Fig. 1. Chromatography of the crude chymosin extract on histidyl-sepharose at room temperature (20°C). Milk clotting activity (☆—☆) is observed only in the second peak. The flow-rate was 30 ml/h. ✱—✱, Absorbance at 280 nm; other details as in the text. Eluents: 1, 0.01 *M* acetate buffer, pH 5.5; 2, 0.01 *M* acetate buffer + 0.5 *M* NaCl, pH 5.5.

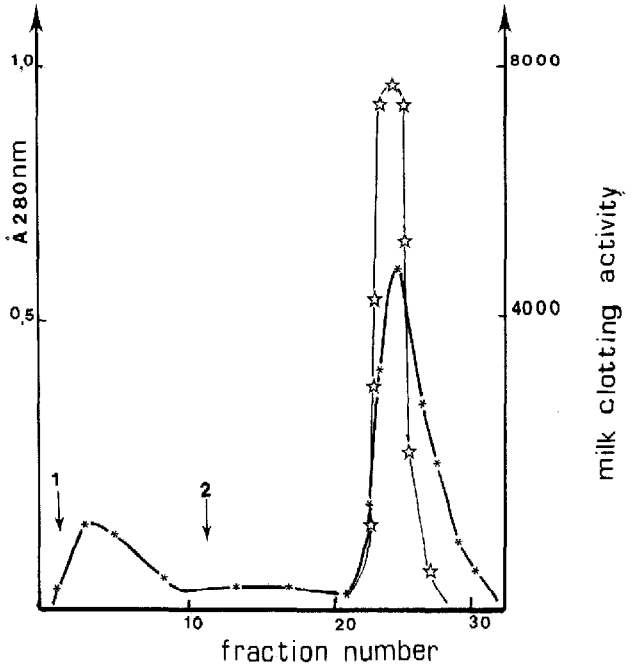
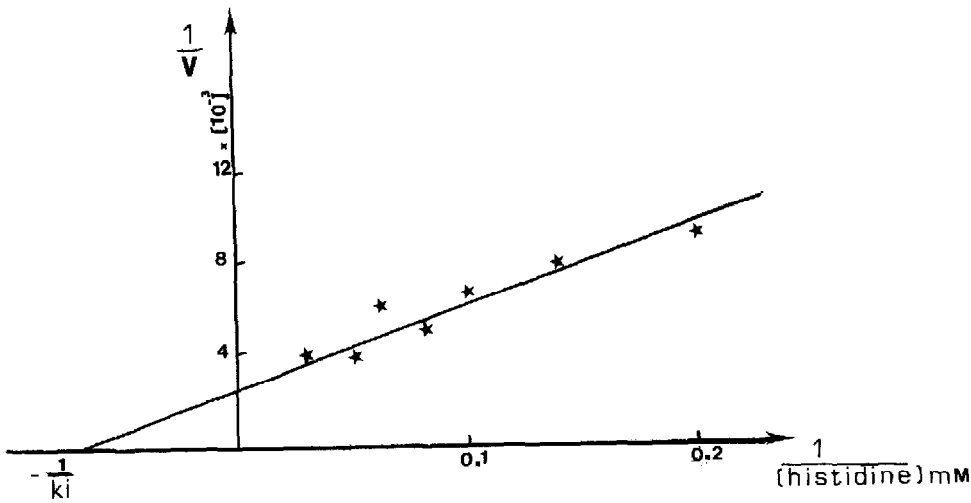


Fig. 2. Chromatography of the crude chymosin extract on histidyl-sepharose at 4°C. Details as in Fig. 1



yield with histidine compared to a 6-fold purification with 60% yield with pepstatin as the affinity ligand. However, the coupled ligand concentration in our experiments is 32 μmol histidine per ml gel, that used in the case of pepstatine was only 1.5–2.0 μmol per ml gel.

Characteristic of the purified chymosin

On gel filtration with a Sephacryl S-200 column the enzyme exhibited a single peak corresponding to a molecular weight of 44,000. Alais[11] reported a molecular weight of 38,000 for calf chymosin as determined by SDS electrophoresis. SDS-PAGE gave a single band for our kid chymosin purified on the histidine column. These results together with those from gel filtration show that the enzyme is a monomer and does not undergo any autoproteolysis under our chromatographic conditions.

The isoelectric point as revealed by isoelectric focusing was at pH 6.0. Three isoenzymes with isoelectric points at pH 4.5, 4.5 and 4.6 respectively have been reported[12].

Proteolytic activity

Proteolytic activity was monitored at pH 5.5 since the enzyme is most stable at this pH. We experienced difficulty in dissolving the casein, solubility being attained only after a long time. A standard assay was carried out with a tyrosine solution of known concentration:

$$\text{Number of units per ml} = \frac{4}{10} \times \text{optical density } K \times N$$

where K = concentration of tyrosine in solution having an optical density of 1.0 and N = dilution factor. One unit of proteolytic activity is the quantity of enzyme per minute which liberates a known quantity of substance which cannot be precipitated

TABLE III

COMPARISON OF PROTEOLYTIC AND MILK CLOTTING ACTIVITIES OF THE ENZYME PURIFIED AT 4 AND 20°C

Conditions (°C)	*n-Fold purification*	*Yield (%)*	*Ratio milk clotting/proteolytic*
4	26	331	1.692
20	18	108	711

by TCA, under the experimental conditions, and which gives the same absorbance as that of 1 mg tyrosine with Folin–Ciocalteau reagent.

Table III gives a comparison of the ratios of the milk clotting activity and proteolytic activity for the enzyme purified at 4°C and that purified at 20°C. The increased specificity of the binding to histidine of the enzyme with milk clotting activity (chymosin) at 4°C is once again revealed by the higher ratio.

CONCLUSIONS

Although the mechanism of specific binding of chymosin to the coupled histidine cannot completely be elucidated in the light of its inhibition constant, we have a highly efficient method for chymosin purification. Moreover, the comparative costs of histidine and pepstatin as affinity ligands for chymosin purification indicate that this new method is promising for industrial applications.

The histidine coupled column seems to be useful for purifying chymosin from the kid, calf and from microbial origins, although with different yields and degrees of purification, and the purified enzymes do not undergo any denaturation or inactivation and are stable.

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