

# Incorporation of isoleucine into protein by a soluble fraction from spermatozoa

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A heat-labile, non-dialyzable factor(s) in soluble fractions from porcine, bull, rabbit and cock spermatozoa was found to incorporate the radioactivity of [ $^{14}\text{C}$ ]isoleucine into a 95°C  $\text{CCl}_3\text{COOH}$ -insoluble fraction. The incorporation required ATP,  $\text{Mg}^{2+}$ , casein and 2-mercaptoethanol. Trypsin and  $\alpha$ -chymotrypsin inhibited the incorporation, while RNase A and DNase I did not. A mixture of 19 amino acids other than isoleucine had no effect on the incorporation. The reaction product was identified as protein. The incorporated moiety was the isoleucyl moiety of isoleucine and it retained a free  $\alpha$ -amino group in the product protein. Some other characteristics of this incorporation are also described.

*Isoleucine      Spermatozoa      Incorporation      105 000  $\times$  g supernatant*

## 1. INTRODUCTION

Since the first description of the incorporation of arginine into a 95°C  $\text{CCl}_3\text{COOH}$ -insoluble fraction (hot trichloroacetic acid-insoluble fraction) by a soluble system [1], 3 similar types of soluble enzymes, which catalyze the incorporations of various amino acids, have been reported [2–9]. These 3 enzymes (arginyl-tRNA:protein arginyl transferase [3], leucyl-, phenylalanyl-, methionyl-tRNA:protein leucyl, phenylalanyl, methionyl transferase [7,8], and glutamyl-tRNA synthetase [9]) catalyze the transfers of aminoacyl moieties from tRNA to the proteins. Therefore, these incorporations require tRNA. On the other hand, the incorporation of methionine which appears not to require tRNA, was also reported [10].

Recently, I observed incorporation of the radioactivity of [ $^{14}\text{C}$ ]isoleucine into a hot trichloroacetic acid-insoluble fraction by 105 000  $\times$  g supernatants from the spermatozoa of various animals, and characterized the incorporation by the supernatant from porcine spermatozoa. These results are described below.

## 2. MATERIALS AND METHODS

Porcine and bull semen was purchased from Kanagawa Livestock Improvement Association, Japan. Rabbit and cock semen was donated from Kanagawa Ken Chikusan Shikenjo, Japan. Spermatozoa of sea urchin (*Pseudocentrotus depressus*) was collected by usual methods. L-[U- $^{14}\text{C}$ ]isoleucine (348 Ci/mol) was from The Radiochemical Centre, England. Bovine casein was from Sanko Junyaku, and was further purified as described [8]. Bovine RNase A, DNase I, trypsin and  $\alpha$ -chymotrypsin were from Sigma. Other materials were commercially available reagent-grade products.

### 2.1. Preparation of 105 000 $\times$ g supernatants from various spermatozoa

Spermatozoa were collected from 100 ml of porcine semen by centrifugation at 20 000  $\times$  g for 10 min, followed by washing twice with 0.9% NaCl at 15°C. All subsequent procedures were carried out at 5°C. After washing, the spermatozoa, about 2 g, were suspended in 2 vols of extraction buffer (20 mM Tris-HCl buffer, pH 7.8, contain-

ing 5 mM magnesium acetate, 0.1 mM EDTA, 100 mM 2-mercaptoethanol, 30 mM KCl and 250 mM sucrose). The suspension thus prepared was homogenized by 10 cycles of sonication (Ohtake Works) at 20 kHz for 30 s. The homogenate was centrifuged at  $20000 \times g$  for 20 min and the resulting supernatant was further centrifuged at  $105000 \times g$  for 90 min. The clear  $105000 \times g$  supernatant was dialyzed overnight against 5 l of 20 mM Tris-HCl buffer, pH 7.8, containing 100 mM 2-mercaptoethanol. The slight turbidity was eliminated by centrifugation. The clear dialysate was referred to as  $105000 \times g$  supernatant from porcine spermatozoa. For the preparation of  $105000 \times g$  supernatants from bull, rabbit, cock and sea urchin spermatozoa, the same conditions were applied except that the weights of starting materials were scaled down. The weights of bull, rabbit, cock and sea urchin were 0.5, 0.4, 1.3 and 0.5 g, respectively.

## 2.2. Assay of the incorporation

The incubation mixture for incorporation of the radioactivity of [ $^{14}\text{C}$ ]isoleucine into hot trichloroacetic acid-insoluble fraction contained 30  $\mu\text{mol}$  Tris-HCl buffer, pH 8.8, 4 nmol L-[U- $^{14}\text{C}$ ]isoleucine (348 Ci/mol), 300 nmol ATP, 1  $\mu\text{mol}$  magnesium chloride, 1 mg casein, 10  $\mu\text{mol}$  2-mercaptoethanol and the  $105000 \times g$  supernatant in a total volume of 100  $\mu\text{l}$ , and the incubation was performed for 30 min at 37°C. Incorporated radioactivities were determined on a 50  $\mu\text{l}$  aliquot as in [11]. Protein was determined according to [12].

## 2.3. Preparation of reaction product

Conditions for the incorporation were applied but with the reaction volume scaled up to 1.8 ml. Separation of hot trichloroacetic acid-insoluble materials from the incubation mixture was performed according to [13]. The hot trichloroacetic acid-insoluble precipitates were dissolved in 1 ml of 1 M NaOH containing 0.1% thioglycolic acid and dialyzed overnight against 2 l of 20 mM Tris-HCl buffer, pH 7.8. The recovery of the incorporated radioactivity was 72.4%. The dialysate was used below as reaction product.

## 2.4. Dinitrophenylation

This was performed according to [14].

## 3. RESULTS

### 3.1. Incorporation by $105000 \times g$ supernatants from various sources

As shown in table 1, the supernatants from porcine, bull, rabbit and cock spermatozoa incorporated the radioactivity of [ $^{14}\text{C}$ ]isoleucine into the hot trichloroacetic acid-insoluble fraction, but that from sea urchin spermatozoa, porcine tissues other than testis, yeast and *Escherichia coli* B did not. The highest level of the activity was found in porcine and the lowest level was in bull. The level of cock was less than 1/6 of that of porcine. Therefore, further studies were carried out on the incorporation catalyzed by the supernatant from porcine spermatozoa.

### 3.2. Requirements for incorporation

The incorporation required ATP,  $\text{Mg}^{2+}$ ,

Table 1  
Incorporation of isoleucine by  $105000 \times g$  supernatants from various sources<sup>a</sup>

Source	Incorporation (cpm)	Specific activity (pmol/min per mg protein)
Spermatozoa		
Porcine	3262	1.32
Bull	1044	1.59
Rabbit	750	1.79
Cock	495	0.24
Sea urchin	38	0.01
Testis <sup>b</sup>	143	0.12
Ovary <sup>b</sup>	17	0.01
Brain <sup>b</sup>	11	0.00
Liver <sup>b</sup>	21	0.01
Spleen <sup>b</sup>	14	0.00
Kidney <sup>b</sup>	0	0.00
Heart <sup>b</sup>	17	0.01
Baker's yeast <sup>c</sup>	37	0.02
<i>Escherichia coli</i> B <sup>c</sup>	29	0.01

<sup>a</sup> Experimental conditions were as described in section 2 using 30  $\mu\text{l}$  supernatant

<sup>b</sup> Conditions for preparation of the supernatants from porcine tissues were the same as for spermatozoa except that homogenizations were carried out with a glass-teflon homogenizer instead of a sonicator

<sup>c</sup> Conditions for preparation of these supernatants were the same as for spermatozoa except that the homogenizations were carried out with a mortar grinder (MRK-RETSCH) instead of a sonicator

Table 2  
Requirements for the incorporation of isoleucine

	Relative activity (%)
Complete <sup>a</sup>	100
– [ <sup>14</sup> C]isoleucine	0
– 105 000 × g supernatant	0
+ the supernatant exposed to 90°C for 1 min, 30 μl	2
– ATP	1
– Mg <sup>2+</sup>	13
– 2-mercaptoethanol	4
– casein	15
+ [ <sup>12</sup> C]isoleucine, 4 nmol	57
+ [ <sup>12</sup> C] 19 amino acids other than isoleucine, 4 nmol each	97
+ tRNA, 300 μg	84
+ trypsin, 18 μg	9
+ chymotrypsin, 18 μg	21
+ DNase I, 20 μg	101
+ RNase A, 20 μg	97

<sup>a</sup> Complete mixture was the same as the incubation mixture of table 1, using the supernatant from porcine spermatozoa

2-mercaptoethanol and casein (table 2). Exposure of the supernatant to 90°C for 1 min resulted in a complete loss of the activity.

### 3.3. Effects of some additions on incorporation

A mixture of 19 amino acids other than isoleucine had practically no effect on incorporation (table 2). This result suggests that the incor-

poration is specific to isoleucine among 20 amino acids. The incorporated radioactivity was reduced almost stoichiometrically when the same amount of [<sup>12</sup>C]isoleucine was added. This eliminated the possibility that radioactive contaminants in the L-[U-<sup>14</sup>C]isoleucine sample were incorporated. Trypsin and α-chymotrypsin inhibited the incorporation, but DNase I and RNase A did not. Addition of tRNA had no effect. These data suggest that protein(s) participate in the incorporation but nucleic acids do not.

### 3.4. Effects of degrading enzymes on the reaction product

As shown in table 3, trypsin and α-chymotrypsin degraded the reaction product, but DNase I and RNase A did not. Accordingly, it can be concluded that isoleucine is incorporated into protein.

### 3.5. Identification of incorporated isoleucine in the reaction product

The reaction product was analyzed by use of the dinitrophenylation method [14]. After ether ex-

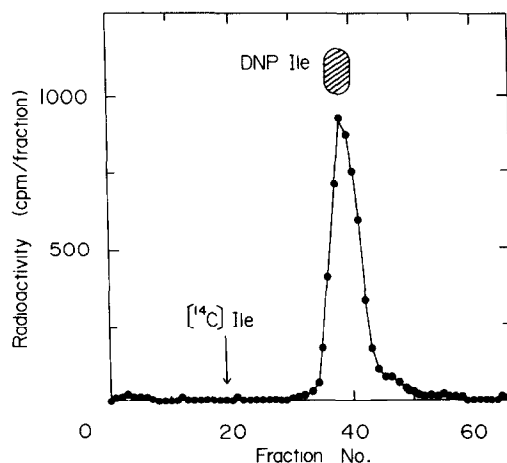


Fig.1. Paper chromatogram obtained from an aliquot (5500 cpm) of the ether extract of the acid hydrolysis of dinitrophenylated product. Paper chromatography was performed for 20 h at room temperature in a descending manner. After chromatography, the paper was cut into 5-mm pieces from the origin to the solvent front, and for each piece the radioactivity was determined. Dinitrophenylisoleucine and L-[U-<sup>14</sup>C]isoleucine were also run on the same sheet. The hatched area indicates the yellow spot of authentic DNP isoleucine and the arrow shows the location of the peak radioactivity of authentic [<sup>14</sup>C]isoleucine.

Table 3

Effects of degrading enzymes on the reaction product<sup>a</sup>

	Relative radioactivity (%)
None	100
Trypsin	3
Chymotrypsin	11
DNase I	93
RNase A	97

<sup>a</sup> Enzymes (20 μg each) were added to the reaction product carrying 3100 cpm radioactivity and incubation was performed in 100 μl of 50 mM Tris-HCl buffer, pH 8.0, for 30 min at 37°C. The radioactivities were determined on 50-μl aliquots according to [11]

traction of the acid hydrolysate of dinitrophenylated product, 77.2% of the radioactivity was recovered in the ether layer. Almost all of the recovered radioactivity migrated as a single spot with an  $R_f$  value of 0.59 on paper chromatography performed using *n*-butanol saturated with 0.1%  $\text{NH}_4\text{OH}$  as the running solvent, and the location of the spot coincided exactly with that of the authentic DNP isoleucine (fig.1). This was further confirmed by another paper chromatography where the running solvent was replaced with 1.5 M sodium phosphate buffer, pH 6.0 (not shown). These results strongly indicate that the isoleucyl moiety of isoleucine was incorporated with retention of the  $\alpha$ -amino group.

#### 4. DISCUSSION

This is the first description of incorporation of the isoleucyl moiety of isoleucine into protein by a soluble system. The incorporation was catalyzed by a soluble, heat-labile and non-dialyzable factor(s) and required ATP,  $\text{Mg}^{2+}$ , casein and 2-mercaptoethanol (table 2).

The incorporation of isoleucine was found in porcine, bull, rabbit and cock spermatozoa but not in sea urchin spermatozoa, porcine tissues other than testis, yeast and *Escherichia coli* B (table 1). A little incorporation by the testis is considered to be attributed to the participation of spermatozoa in testis. From these data, this incorporation of isoleucine is considered to be peculiar to the spermatozoa of vertebrates.

Isoleucine was incorporated into protein (table 3). The reaction site on protein is not yet clear. However, the carboxyl group is possibly excluded because the incorporated isoleucyl moiety retained the  $\alpha$ -amino group (fig.1). Further studies on the reaction site are now underway.

RNase A did not inhibit the incorporation and tRNA was not required (table 2). These data exclude the possibility that this incorporation proceeds via isoleucyl-tRNA. Therefore, this incorporating activity is considered to be different from aminoacyl-tRNA:protein aminoacyl transferase activities, which require tRNA [3,7-9]. This incorporation of isoleucine may be similar to that of methionine [10], which appears not to require tRNA.

Levels of the incorporation of isoleucine in the

supernatant were relatively small as compared with that by arginyl-tRNA:protein arginyl transferase. For instance, the specific activity of the supernatant from porcine spermatozoa was approx. 1/7 of that for incorporation of arginine by the supernatant from yeast [4]. However, further increment in the observed activity might be achievable because better (real) acceptor proteins of isoleucine might exist. Incorporation might well be dramatically increased by the addition of a better acceptor protein, just as in the case of arginyl-tRNA:protein arginyl transferase where several-fold increases of incorporation were observed upon the addition of specific acceptor protein [4].

I do not know whether the real acceptor protein may exist in spermatozoa itself or corresponding eggs. If the acceptor protein were in eggs, this incorporation would be considered to participate strongly in the fertilization.

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